INFLUENCE OF THE FEMALE REPRODUCTIVE TRACT ON THE MOTILITY AND MORPHOLOGICAL CHARACTERISTICS OF RAM SPERMATOZOA

Thesis submitted by

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DECLARATION

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ABSTRACT

In mammals, millions of spermatozoa are deposited in the posterior female reproductive tract but only a few hundred reach the oviducts and from these only one will fertilise an oocyte in a mono-ovulatory species. Investigating why so few spermatozoa reach the oviduct and what was so special about these spermatozoa was the central theme to the studies reported in this thesis. The studies were conducted in the facilities of the Biomedical and Tropical Veterinary Science precinct, James Cook University and used Merino sheep as the model.

In initial studies, semen was collected from rams by electroejaculation and it was demonstrated that not only did ram spermatozoa in undiluted semen have a limited life span but that were differences between rams. Some rams had spermatozoa that survived for less than six hours whereas in others, spermatozoa survived for 30 hours. These differences between rams were not present in semen diluted in Tyroide's-albumin-lactate-pyruvate (TALP) medium. Nearly all spermatozoa in freshly ejaculated semen were uncapacitated but after 12 hours incubation in Hepes-synthetic oviduct fluid (HSOF), 70% were capacitated.

Baseline information on the detailed motility and movement characteristics was determined with a computer-aided semen analyzer (CASA). The results demonstrated that there is a heterogenous population of spermatozoa in a semen sample and that some rams had spermatozoa that had a significantly larger head area than others. This result was supported in later studies by manual measurements of the width and length of heads of spermatozoa.

Semen was collected each month for 13 months from a group of six rams. A range of measurements of the semen was made including volume, colour and velocity and movement characteristics of spermatozoa as determined by CASA. These data were correlated with meteorological data. The quality of semen was significantly influenced by the mean daily maximum temperature and hours of bright sunshine with the months January to March being the times when ram semen was of poorest quality.

Samples of spermatozoa were collected from a range of sites in the reproductive tract of naturally-mated ewes at 3, 6 and 24 hours after mating. The spermatozoa were examined for detailed velocity and movement characteristics, capacitation status and dimensions of spermatozoa. A surprising result was the low and variable percentage (range 2%-22%) of motile spermatozoa in the uterus particularly at 3 and 6 hours after mating declining to a mean of 2.7% 24 hours after mating. No difference in the velocity and movement characteristics of spermatozoa between the anterior and posterior reproductive tract could be identified.

However, two important and interesting results were found. There was evidence that the ovary bearing the pre-ovulatory follicle or corpus haemorrhagicum influenced the distribution of spermatozoa as significantly more spermatozoa were found in the mid and anterior ipsilateral uterine horn and oviducts than the contralateral side 24 hours after mating. The same occurred 6 hours after mating except there was only a non significant trend for the uterine horns. The second important finding was that the spermatozoa in the oviducts had a significantly smaller head than elsewhere in the reproductive tract.

Analysis of the capacitation status of spermatozoa demonstrated that spermatozoa can undergo capacitation and the acrosome reaction in all sites of the reproductive tract and by 24 hours after mating most spermatozoa were capacitated and acrosome-reacted.

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AC	anterior cervix
ALH	amplitude of lateral head displacement
AMP	ampulla
ANOVA	analysis of variance
AR	acrosome reaction
AV	anterior vagina
AUL	anterior uterus-left
AUR	anterior uterus-right
BCF	beat cross frequency
BSA	bovine serum albumin
BU	body of uterus
C	Celsius
CAI	capacitated acrosome-intact
cAMP	cyclic adenosine monophosphate
CAR	capacitated acrosome-reacted
CASA	computer-aided semen analysis
CIDR	controlled internal drug release
CON	contralateral
CTC	chlortetracycline
G	gauge
g	gram
h	hour
Hepes	N-2-hydroxyethilpiperazine-N'-2ethanesulphonic acid
HSOF	hepes-buffered synthetic oviduct fluid
Hz	Hertz
IPS	ipsilateral
IU	international unit
IVOS	integrated visual optical system
LIN	linearity
LVS	low VAP cut off
LVV	low VSL cut off
NM	natural mating
MC	midcervix
mg	milligram
mM	millimol
MPA	medroxy progesterone acetate
MVV	medium VAP cut off
MUL	miduterus-left
MUR	miduterus-right
O	oestrus
ODB	oestradiol benzoate
P	progesterone
PBS	phosphate buffered saline
PGE	prostaglandin E
$PGF_{2\alpha}$	prostaglandin F2 alpha
pH	potential hydrogen
PMSG	pregnant mare serum gonadotrophin

s	synchronized
SEM	standard error of the mean
So	threshold straightness
STR	straightness
TALP	Tyrode's albumin-lactate-pyruvate
µg	microgram
µl	microlitre
µm	micrometer
µmsq	micrometer square
UTJ	utero-tubal junction
us	unsynchronized
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight-line velocity
v/v	volume/volume

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Artificial insemination is used world-wide in livestock production in particular the dairy industry as well as in the reproductive management of companion animals and horses and the treatment of some forms of infertility in humans. In mammals, millions of sperm are deposited in the female reproductive tract either at natural mating or artificial insemination in order to fertilise only one oocyte in the case of mono-ovulating species. Of the millions of spermatozoa inseminated, probably only about a thousand reach the isthmus of the oviduct and even fewer reach the ampullary-isthmic junction at the time of fertilisation (Anderson, 1991).

The female reproductive tract is a hostile environment for spermatozoa. When spermatozoa are recovered from the female reproductive tract, many are found to be dead or damaged but some are vigourously motile (Bedford, 1982). In addition to the hostile environment, spermatozoa have to negotiate the considerable anatomical barriers of the mucus filled folds of the cervix and the glands of the endometrium in order to reach the relative safety of the oviducts. Why the female reproductive tract should be so hostile to spermatozoa is not clear from a biological stance but from an evolutionary viewpoint, the hostility may be related to the role of the female in spermatozoa competition that exists in some species (Greeff and Parker, 2000).

Because of the critical role of spermatozoa in reproductive success, the biology and function of spermatozoa in the female reproductive tract has received considerable attention (Gomez *et al*., 1997; Cross, 1998; Lapointe *et al*., 1998; Boquest *et al*., 1999; Boquest and Summers, 1999; Ellington *et al*., 1999; Fazeli *et al*., 1999; Si and Okuno, 1999; Thundathil *et al*., 1999; Al-Hijji *et al*., 2000; Barrios *et al*., 2000; Kaul *et al*., 2001).

An example of where a detailed understanding of what happens to spermatozoa in the female reproductive tract is important is the need to maximise the chances of

conception with artificial insemination with minimal numbers of spermatozoa particularly when new techniques such as X- and Y- sperm separation are used (Hollinshead *et al*., 2002).

There is much to be understood about the normal physiological, cellular and biochemical events that occur in the process of migration of spermatozoa from the site of deposition of semen to the site of fertilisation. Of particular interest is the question as to what is different about spermatozoa that reach the site of fertilisation relative to those spermatozoa that remain in other sites of the female reproductive tract. It might be that the female reproductive tract is able to select a population of spermatozoa from the ejaculate and that it is this group of spermatozoa that make their way to the site of fertilisation. Considering that the ejaculate consists of a heterogenous population of spermatozoa (Thurston *et al.,* 1999), a method to select the best spermatozoa from the heterogenous population would confer an advantage in terms of reproductive success. The concept that the female can influence the fate of spermatozoa in the female reproductive tract has precedence elsewhere in the animal kingdom with respect to sperm competition after mating by different males (eg. in birds). This physiological control over the paternity of their offspring is referred to as cryptic female choice (reviewed by Birkhead, 1995).

1.2 Working Hypothesis

The hypothesis tested in this research project was that the female reproductive tract selects those spermatozoa from an inseminate that have optimal motility and morphological characteristics necessary to achieve fertilisation. The hypothesis was tested using Merino sheep as the experimental model.

1.3 Aims and Objectives

The aims and objectives of the studies described in this thesis were:

- To evaluate *in vitro* capacitation and motility characteristics of ram spermatozoa.

- To evaluate any variation in motility parameters of spermatozoa within and between Merino rams as determined by computer-aided semen analysis.
- To define the distribution, motility and morphological characteristics of ram spermatozoa at various sites in the reproductive tract of Merino ewes at defined times after mating.
- To examine any local effects of the ovary containing the pre-ovulatory follicle or corpus haemorrhagicum on the motility characteristics of spermatozoa in the anterior uterine horn and oviduct.
- To determine where in the reproductive tract of the ewes that capacitation occurs and the influence of the side of ovulation on the distribution of capacitated spermatozoa.

CHAPTER 2

REVIEW OF THE LITERATURE

Very few spermatozoa, as a proportion of those in an ejaculate, reach the oviducts. Anderson (1991) reported that in the ewe about 600 spermatozoa reach the site of fertilisation at each isthmic-ampullary junction. Assuming a single ejaculate of 0.8 ml of ram semen of 0.8 ml with a sperm concentration of 4.5 x 10^9/ml and 90% motile spermatozoa (3.24 x 10^9 motile spermatozoa) it means that greater than 99.9% of motile spermatozoa did not arrive at the site of fertilisation. The question is where do all the spermatozoa go? What follows in this Chapter is a review of the scientific literature on the fate of spermatozoa in the female reproductive tract after natural or artificial insemination with particular emphasis on the sheep.

2.1 Female Reproductive Tract

2.1.1 Vagina

While there are exceptions, such as the pig and mare, in many species the first part of the female reproductive tract that spermatozoa will encounter will be the vagina and cervix. The vaginal fluid is composed primarily of a transudate derived through the vaginal wall, mixed with vulval secretions from sebaceous glands and contaminated with cervical mucus, endometrial and oviductal fluids and exfoliated cells of the vaginal epithelium (Hafez, 1980).

Vaginal mucus is acidic and therefore hostile to spermatozoa. In ewes the vaginal mucus at oestrus is pH 5.7 (O'Shea and Murdoch, 1978), whereas in humans, the vaginal pH is 4.0 to 6.0 (Ramsey *et al*., 2002). The acidicity of the vaginal mucus is counteracted by the mild alkalinity of semen (Mann and Lutwak-Mann, 1981) to provide a near neutral and therefore less hostile environment for spermatozoa. Nevertheless, spermatozoa do not survive for long periods in the vagina with early studies indicating that progressive motility of spermatozoa could be maintained for only six hours in Merino ewes (Quinlan *et al.,* 1932).

Vaginal mucous impedance appears to be primarily controlled by progesterone, but it also changes in response to shifts in the oestradiol: progesterone ratio when progesterone concentrations are low. Impedometric characteristics of the vaginal mucosa in cyclic ewes are an indicator of serum concentrations of progesterone and oestradiol: progesterone ratios during the terminal stage of the oestrous cycle (Bartlewski *et al*., 1999). Similar studies in cattle have identified the low impedence characteristics of vaginal mucus at oestrus (Rutllant *et al.,* 1999). Traces of elements such as calcium, copper, chromium, cadmodium, potassium, magnesium, zinc, mercury, phosphates and also bicarbonates are present in vaginal, cervical and uterine mucus (Harvey *et al*., 1960).

2.1.2 Cervix

The cervix is a sphincter-like structure that projects caudally into the vagina and is characterized by a thick wall and constricted lumen (Hafez, 1980). Although the structure of the cervix differs in detail among farm mammals, the cervical canal is characterised by various prominences. The cervix is the first barrier that spermatozoa have to overcome in those species where the ejaculate is deposited in the vagina. Unlike other domesticated animals, the cervix of the sheep is highly contorted and one assumes would be a significant barrier to spermatozoa. The cervix is a significant barrier to frozen-thawed ram spermatozoa and because the anatomical features make it very difficult to pass a insemination pipette through the cervix, the technique of laparoscopic intra-uterine insemination of frozen-thawed spermatozoa is used to bypass the cervix (McKelvey *et al*., 1985).

Cervical mucus is a visco-elastic substance. Goblet cells in the cervical epithelium secrete mucin granules which swell and become transformed, forming a hydrogel matrix of interconnected macromolecules with low viscosity mucus plasma in their interstices (Katz *et al*., 1989). Cervical mucus consists of macromolecules of mucin of epithelial origin, which are composed of glycoproteins that contain about 25% amino acid and 75% carbohydrate. The mucin is composed of a long, continuous polypeptide chain with numerous oligosaccharide side chains. The carbohydrate portion is made of galactose, glucosamine, fucose and sialic acid (Hafez, 1980).

Glucose and fructose concentrations in the cervical mucus at normal oestrus in the cow have been reported to be 7.3 mg/100 ml and 4.9 µg/ml respectively (Tsiligianni *et al*., 2001). The carbohydrate composition of the glycoproteins plays a key role in enabling spermatozoa to readily migrate through cervical mucus (Morales *et al*., 1993). Viscosity and crystallization of cervical mucus is related to the time of ovulation and it has been noted that in bovine cervical mucus, viscosity was lower and crystallization was higher in normal oestrus compared to induced oestrus (Tsiligianni *et al*. 2002).

Optimal changes in cervical mucus properties, such as an increase in quantity, viscosity, ferning and pH and decrease in viscosity and cell content, occur during the preovulatory period (Hafez, 1980). In humans, the pH of ectocervical mucus is 6.91 while the pH of endocervical mucus is 7.09 (Correa *et al*., 2001). The cervical mucus of cows in oestrus has a pH of about 7, a viscosity of 23.2 mm H_2O and spinnbarkeit of 72 mm (Verberckmoes *et al*., 2002).

The crypts of cervix are regarded as the largest reservoir of spermatozoa in humans and ruminants but the question arises as to whether there is an active selection process occurring in the cervix. Which spermatozoa pass through the cervix and which are retained within the cervical crypts? What determines when and which spermatozoa are released from the cervical crypts? One current interpretation is that spermatozoa that eventually make their way to the oviducts and fertilise an oocyte pass through the central diluted mucus of the cervix into the uterus rather than become closely associated with the epithelium of the cervical crypts (see Hunter, 2001). The literature does not contain any information on the exact nature of interaction between cervical epithelium and spermatozoa and if in fact this interaction has any biological significance.

The concept of the cervix acting as a reservoir of spermatozoa is based on the observations of large numbers of spermatozoa being present for some time in the cervix (Mattner, 1966) as well being able to maintain motility in the cervix for at least 48 hours after insemination, at least in humans (Hanson *et al.,* 1982). However, whether the cervix supplies spermatozoa to progress to the oviducts for many hours

after mating is unknown because there has been no direct observation of spermatozoa in the oviducts that previously had been stored in cervix.

2.1.3 Uterus

The uterus consists of a thin outer layer, the perimetrium, a thick myometrium composed of inner circular and outer longitudinal smooth muscle layers, and an inner layer, the endometrium. The changes that occur in the endometrium during the oestrous cycle prepare the uterus to receive the blastocyst and therefore play a major role in the reproductive process (Hafez, 1980). The volume and biochemical composition of the uterine fluid shows consistent variation throughout the oestrous cycle. In sheep, the volume of the fluid in the uterus exceeds that of the oviduct during oestrus, whereas during the luteal phase the fluid in the uterus is much less than at oestrus (Perkins *et al*., 1965). The endometrial fluid contains mainly serum proteins but also small amounts of uterine-specific proteins. The ratio and amounts of these proteins vary according to the stage of the oestrous cycle (Hafez, 1980).

2.1.4 Oviduct

The wall of the oviduct is composed of mucosa, muscularis and serosa. The oviductal mucosa is made of primary, secondary and tertiary folds. The mucosa in the ampulla is thrown into high, branched folds that decrease in height toward the isthmus, and become low ridges in the uterotubal junction. The ciliated cells of the oviductal mucosa have slender motile cilia (kinocilia) that extend into the lumen (Hafez, 1980). Secretory cells release their products into the oviduct lumen through pores or high protrusions of cells which become detached, decomposed and form part of the secretion (Cigankova *et al.,* 1996).

The oviductal and uterine musculature of the ewe reaches a peak in sensitivity to physiological concentrations of oxytocin at oestrus. When combined with information on oxytocin receptor populations and endogenous circulating concentrations, it suggests that endogenous oxytocin could influence oviduct and myometrial activity (Gilbert *et al*., 1992). In cattle, the concentration of a number of hormones including prostaglandin E, prostaglandin $F_{2\alpha}$ and endothelin are higher in

the ipsilateral than contralateral oviduct at oestrus and these may influence the activity of the oviductal musculature (Wijayagunawardane *et al.,* 1998). While the highest concentration of progesterone is found during the luteal phase in the oviduct ipsilateral to the functional corpus luteum, oviductal oxytocin was unchanged throughout the cycle.

In the infundibulum of cows, there are cul-de-sacs that open toward the ovary, while cul-de-sacs present in the caudal isthmus and in the utero-tubal junction open towards the uterus. Marked variations have been observed in the oviductal epithelium depending on the oviduct segment, basal or apical areas of the folds, and phase of the oestrous cycle (Yaniz *et al*., 2000).

The fluids of the female reproductive tract, in particular the oviductal fluids at the follicular stage, provide a suitable environment for the maintenance of the viability and motility of spermatozoa. There is evidence that it is the secretory products of oviductal epithelial cells that play an important role in sustaining both the viability and motility of spermatozoa (Abe *et al*., 1995). Oviduct-specific glycoprotein has been demonstrated to be the major secretory glycoprotein and to be expressed exclusively by the oviduct. This protein, under oestrogen control during the follicular phase and during oestrus is conserved across all species examined. Oviduct-specific glycoproteins affect capacitation, motility and viability of spermatozoa (Buhi, 2002).

2.2 Spermatozoa Reservoirs and the Life Span of Spermatozoa in the Female Reproductive Tract

2.2.1 Spermatozoa reservoir

In ruminants, minor cervical folds, commonly called crypts, appear to temporarily store spermatozoa for a short interval of time between insemination and fertilisation (Quinlivan and Robinson, 1969; Wirgin, 1985). However, as there is possibly only a gradual progression of viable spermatozoa from the cervix to the oviduct (see section 2.1.2), the isthmus rather than the cervix is regarded as the main functional spermatozoa reservoir near the time of ovulation, with some form of peri-ovulatory

programming of release of spermatozoa from the isthmus (Hunter *et al.,* 1980; Hunter *et al.,* 1983; Hunter, 1984).

Follicular fluid passing down the ampulla at ovulation is not considered to be the physiological stimulus for an initial, tightly-controlled release of spermatozoa from epithelial binding in the caudal isthmus. Indeed, because such spermatozoa activation commences shortly before ovulation, a locally transmitted ovarian programming via relatively high concentrations of follicular hormones remains the favored model (Hunter *et al*., 1980). Although pre-ovulatory progesterone is considered to be the coordinating steroid of increasing influence in these pre-fertilisation events, it is proposed that its effect is modulated in the endosalpinx by mobilisation of Ca^{2+} ions into the discrete population of bound spermatozoa (Hunter *et al*., 1999). In cattle, near the time of ovulation, numerous spermatozoa are found in the periphery of the caudal isthmus within pockets of basal interfold areas, as well as within pockets and cul-de-sacs of the tubo-uterine junction. Individual spermatozoa were also observed in peripheral areas of the ampullary-isthmic junction and ampulla (Yaniz *et al*., 2000). Recent evidence indicates that spermatozoa are trapped in the reservoir by binding to specific carbohydrate moieties on the surface of the mucosal epithelium of the oviduct. A bovine seminal plasma protein has been identified that associates with spermatozoa and confers on them the capacity to bind to the carbohydrate moiety on oviductal epithelium (Suarez, 2002).

In the oviducts, the reservoir of spermatozoa may serve three functions. First, it may prevent polyspermic fertilisation by allowing only a few spermatozoa at a time to reach the oocyte in the ampulla. Second, the oviductal reservoir may maintain the fertility of spermatozoa until the oocyte is released and enters the oviduct. Third, the physiological state of spermatozoa, specifically the processes of capacitation and motility hyperactivation, may be regulated within the reservoir to ensure that spermatozoa are in the proper state when ovulation occurs (Suarez, 2002).

2.2.2 Life-span of spermatozoa within the female reproductive tract

The longevity of spermatozoa in the female reproductive tract of the ewe can be influenced by factors such as the quality of spermatozoa, whether the semen is fresh or frozen-thawed and the time of insemination relative to the onset of oestrus. The longevity of frozen-thawed bull spermatozoa in the female reproductive tract is about half that of fresh spermatozoa (12-24 hours) (Gordon, 1994) and a similar reduction in longevity is inferred from studies in sheep (Gillan *et al*., 1999).

There is wide variation among animal species in the longevity of spermatozoa in the female reproductive tract. Fertilisation in horses in which oestrus can last up to seven days, can occur six days after coitus (Burkhardt, 1949). In dogs, oestrus normally lasts for about nine days (Olsen and Nett, 1986) and spermatozoa can remain viable in the tract for three to six days (Doak *et al*., 1967). A really extreme example of survival are some species of bat where spermatozoa maintain fertilising capacity for 135 days (Hogarth, 1978). Hunter (1988) has provided information (Table 2.1) on the longevity of spermatozoa and oocytes in the female reproductive tract of various mammalian species but what is not clear is from what sites of the reproductive tract this information refers to. Viable spermatozoa at 48 hours after insemination in the cervix of the ewe does not necessary mean that these spermatozoa can progress to the oviducts and fertilize. On the other hand, viable spermatozoa at 48 hours in the isthmus could have a higher probability of fertilising a recently ovulated oocyte.

Table 2.1 The life-span of gametes in the female reproductive tract, representing the period during which they are able to achieve normal fertilisation and subsequent cleavage (from Hunter, 1988).

Species	Spermatozoa (hours)	Oocytes (hours)
Sheep	24 - 48	10 – 15
Cattle	24 - 48	10 – 12
Horse	140	8 – 10
Pig	24 - 42	8 – 12
Human	24 - 72	8 – 10

It has been observed in a number of species that some spermatozoa reach the oviducts within 30 minutes of mating (Anderson, 1991). The evidence from studies such as those by Hunter and Nichol (1983) using ewes indicates that these spermatozoa have a limited life span and do not participate in fertilisation. A second wave of spermatozoa reach the oviducts 6-8 hours later and it is from this population that the fertilising spermatozoa are derived (see Section 2.3.2).

2.3 Transport of Spermatozoa in the Female Reproductive Tract

Spermatozoa are transported from the site of ejaculation to the oviducts by a variety of actions including smooth muscle contractions of the reproductive tract, ciliary beats of epithelial cells, fluid currents and flagellar activity of spermatozoa (Hunter, 1988). The mechanical stimulus of mating may also enhance uterine contractions and spermatozoa distribution (Overstreet and Katz, 1990). The dynamic interactions that occur between functional spermatozoa and the luminal fluids and epithelial surfaces of the female genital tract during transit and storage enhance spermatozoa survival and regulate spermatozoa function in the female. The universal nature of this interaction highlights it as a key component of the spermatozoa transport process (Scott, 2000).

A common feature in all mammals that have been studied is that there is a rapid transport of spermatozoa to the oviducts normally within minutes of mating (Anderson, 1991). The swimming rate of mammalian spermatozoa is less than 8 mm per minute (Hogarth, 1978) and is inadequate to explain the rapid appearance of spermatozoa in the oviducts. The only obvious explanation is that spermatozoa reach the oviducts through contractions of the uterus (see Section 2.4). This view is supported by the observation that inert particles deposited in the vagina of ewes in oestrus are found in the oviduct within 15 minutes (Hogarth, 1978).

It appears that the rapidly transported spermatozoa are not involved with fertilisation. The work of Overstreet and Cooper (1978) in the rabbit showed that most of the rapidly transported spermatozoa had disrupted acrosomes and many also had separated heads from tails. The assumption is that this occurs in other species. Circumstantial evidence from the oviduct ligation work by Hunter *et al.* (1980)

indicates rapid transport disrupts the structure of spermatozoa in sheep. Whether a rapid appearance of spermatozoa in the oviducts occurs after artificial insemination into the vagina seems not to have been defined. As discussed later (Section 2.3.2), a second wave of spermatozoa reaches the oviduct several hours after mating and it is from this population that fertilizing spermatozoa are derived.

2.3.1 Transport of spermatozoa into and through the cervix

After ejaculation into the vagina, a pool of concentrated spermatozoa in semen bathes the external os of the cervix and spermatozoa enter the mucus-filled cervical canal largely as a result of their own motility. Despite the major role of motility of spermatozoa on colonising the cervix, the process is quite rapid at least for a small population of competent spermatozoa. Smooth muscle contraction of vaginal and cervical walls probably also contributes to the process (Bedford, 1971; Overstreet and Cooper, 1978; Hunter, 1988).

In ewes, it has been demonstrated that the greatest motility of the cervix occurs during the preovulatory period, an activity that corresponds with contractions of the uterus (Garcia-Villar *et al*., 1982). There is also a suggestion that cervical inflammation induced by insemination (see Section 2.8) plays a role in facilitating the transport of spermatozoa across the cervix by inducing cervical dilation via the action of interleukin-8 (Mitchell *et al*., 2002).

The exact mechanism for the transport of spermatozoa from the minor cervical folds of the ovine cervix to the uterus is not known. Previous studies indicate that the spermatozoa generally are not oriented parallel to the longitudinal axis of the cervix, exhibit no consistent associations with the cervical cilia and do not lie in any well defined channels formed by the cervical secretions (Wirgin,1985). The majority of spermatozoa are found as isolated aggregations that lie in or near the shallow crypts of the cervix. The vast numbers of spermatozoa in these aggregations and the lack of any common orientation suggest that some form of external stimulus, such as cervical contractions, might be responsible for the initial mass movement and distribution of spermatozoa in the cervix of the ewe (Wirgin, 1985).

In ruminants, the cervical mucus forms a complete barrier to seminal plasma (Katz *et al.*, 1989). A greater number of spermatozoa were found in the cervices of ewes inseminated with fresh semen than in the cervices of ewes inseminated with killed spermatozoa at 0.5 and 4 h after insemination. In the ewes inseminated with fresh semen, spermatozoa were between the cervical villi or within the cervical glands at 0.5 and 4 h, respectively. However, spermatozoa were found only in the central area of the cervical lumen in the ewes inseminated with killed spermatozoa. The passage of spermatozoa through the cervical mucus to the cervical mucosa was affected by their own motility (Mattner and Braden, 1969b).

In pigs, spermatozoa have to be transported from the cervical end to the tubal end of the uterine horn after mating or artificial insemination. The transport of semen through the horns is probably a passive process driven by the flow of intrauterine fluid containing spermatozoa, due to gravitational force, movement of the sow and uterine contraction (Scott, 2000).

The importance of the efficiency of migration through cervical mucus and colonisation of the oviduct and fertilising ability was recently demonstrated in goats (Cox *et al*., 2002). Males with a low migration rate through cervical mucus *in vitro* had a lower oviduct colonisation and fertilising ability than those with a high cervical migration rate.

2.3.2 Transport of spermatozoa in the uterus and oviduct

The transport of spermatozoa from the site of deposition of the ejaculate to the site of fertilisation at the isthmic-ampullary junction is thought to be a combination of both passive and active transport processes. The passive part of transport of spermatozoa is probably due to the flow of fluid caused by gravity and by the contractile movement of the uterine horn. Some of the luminal and glandular epithelium of the endometrium are ciliated and cilia may also have a role in movement of spermatozoa (Bacha and Wood, 1990). The literature on the significance of uterine contractility on fertilisation rates is confusing but recent information in the pig suggests that a minimum level of uterine contractility at about the time of insemination appears to be important for the rapid transport of spermatozoa to the anterior ends of the uterine

horns (Langendijk *et al*., 2002). Active transport of spermatozoa via the action of flagellar movement is probably important for migration of spermatozoa from the anterior uterine horn into the oviducts.

The number of spermatozoa in the uterus of the sow decreased to a few percent of the number of inseminated spermatozoa in a few hours after insemination, reaching 0.1% at 24 hours after insemination (First *et al*., 1968; Pursel *et al*., 1978). At the same time the number of spermatozoa in the oviducts was maintained and increased by ongoing migration from the uterine horns during the first 24 hours after insemination (Rigby, 1966; Pursel *et al*., 1978). Establishment of a spermatozoa population in the oviducts occurs rapidly after insemination, as spermatozoa were observed in the oviducts of the sow as early as 5-15 minutes after insemination (First *et al*., 1968; Baker and Degen, 1972).

As discussed previously spermatozoa are transported to the oviducts of sheep within a few minutes after mating, but these spermatozoa probably do not fertilise any ova (Hunter *et al.,* 1980; Hawk, 1983). A second wave of spermatozoa reach the oviduct 6 to 8 hours after mating in sheep and cattle (Hunter *et al*., 1980; Hunter and Nicol, 1983) and it is from this population that the fertilising spermatozoa are derived. Viable spermatozoa are apparently sequestered in the caudal isthmus for as long as 17-18 hours, until shortly before ovulation, when a local transfer of ovarian follicular hormones is thought to facilitate their redistribution (Hunter and Nichol, 1983).

Spermatozoa do not always migrate to the oviducts. When spermatozoa were deposited in one uterine horn of heifers there was significant transuterine migration and spermatozoa were recovered from all parts of the reproductive tract and the contralateral side (Larson, 1986). Similar observations have been made by other workers in pigs (Viring *et al*., 1980) and cattle (Hawk and Tanabe, 1986).

2.4 Contractions of the Female Reproductive Tract and Spermatozoa Transport

Observations in a number of mammalian species have shown that there are uterine contractions in the female at the time of oestrus. While it seems likely that

contractions of the uterus propel the population of spermatozoa that are found within the oviducts within minutes of insemination, it is generally accepted that these spermatozoa soon die and do not fertilise (Hawk, 1983). How important are uterine contractions on moving the fertilising population of spermatozoa is not entirely clear. In the oestrous ewe, 58% of the uterine contractions moved towards the oviduct, while 16% moved towards the cervix. In contrast, however when a plastic spiral was placed in the lumen of one horn only 17% of the contractions moved toward the oviduct and 70% of the contractions moved towards the cervix (Brinsfild and Hawk, 1969). Similar observations have been made in cattle whereby the greatest number of uterine contractions was observed at oestrus with some contractions moving toward the oviducts during the preovulatory period (Rodrigues-Martinez *et al*., 1987; Al-Eknah and Noakes, 1989). Flagellation of spermatozoa is probably required for spermatozoa to enter the folds of the cervix, and flagellation may be helpful or essential for spermatozoa to pass through the uterotubal junction, move from the isthmus to the ampulla, and penetrate ova (Hawk, 1987).

The role of classical neurotransmitters including acetylcholine and norepinephrine in regulating myometrial contractility has been well studied. In rats, the magnitude and nature of uterine contractility in response to activation of neurokinin receptors depends upon the hormonal environment (Hamlin *et al*., 2000). In humans, uterine contraction changes throughout the menstrual cycle as assessed by ultrasound and intrauterine pressure measurements emphasize the hormonal dependence of uterine contractility. Although uterine contraction patterns favouring spermatozoa transport appear to be regulated by oestradiol-17β, uterine quiescence and the dominance of convergent uterine contractions prevailing at the time of implantation are linked to elevated progesterone concentration (Buletti *et al*., 2000). Wijayagunawardane *et al.* (2001) suggested that the periovulatory LH surge, together with increasing oestradiol-17ß levels from the Graafian follicle and basal progesterone from the regressing corpus luteum stimulate maximum oviductal production of prostaglandin and endothelin and this causes oviduct contractions to enable rapid transport of gametes. Oxytocin released from the newly-formed corpus luteum may block these mechanisms, and cause slow contractions to transport the embryo to the uterus.

The question does arise as to whether the motility of the uterus directly moves spermatozoa forward to the utero-tubal junction and then into the isthmus or whether the uterine contractions act as a mixing process that indirectly help spermatozoa to move under their own propulsion.

The adrenergic stimulation of powerful, pre-ovulatory waves of contraction in the isthmus may also act to provoke capacitation and hyperactivation of ram spermatozoa (Hunter and Nichol, 1983). Contractile movement of the oviduct, beating of the epithelial cilia and motility of spermatozoa are all regarded as contributing to the transport of the spermatozoa from the isthmus to the ampulla (Yanagimachi, 1994).

2.5 Influence of Seminal Plasma and Hormones on Transport of Spermatozoa

2.5.1 Influence of seminal plasma

Biochemical constituents of seminal plasma, such as prostaglandins, can stimulate smooth muscle activity of the female reproductive tract and thereby assist the distribution of semen or spermatozoa within the tract (Harper, 1994). Einarson and Viring (1973) reported that a greater number of boar spermatozoa reached the oviducts in four hours when spermatozoa were thawed in seminal plasma compared with a buffer solution. Seminal plasma appears to have an important function in the interaction of spermatozoa with the uterus. In the rabbit, immotile spermatozoa did not reach the oviducts when they were suspended in saline but when immotile spermatozoa were suspended in seminal plasma for artificial insemination, rapid spermatozoa transport to the oviducts was observed (Overstreet and Tom, 1982). Seminal plasma and spermatozoa have several effects on the uterus, probably provoking contraction of the uterus (Katila, 2001).

2.5.2 Hormonal influences

Pharmacological agents that affect myometrial activity have been shown to alter spermatozoa transport in females in oestrus. In a classic experiment, Hawk *et al*. (1982b) injected female rabbits with alpha-adrenoceptor agonists (phenylephrine and ergononine) and found that the number of spermatozoa reaching the oviducts was significantly increased. Acetylcholine injected intra-muscularly into rabbit does immediately after natural mating, significantly increased the number of spermatozoa recovered one hour later from the vagina and cervix but not from the oviducts. Acetylcholine caused immediate increases in the frequency and amplitude of uterine contractions whereas injection of oestradiol one hour before artificial insemination increased the number of spermatozoa recovered from the oviduct, uterus, cervix and vagina at 2.5 hours after insemination. Oestradiol caused little if any detectable increased retention and transport of spermatozoa (Hawk *et al*., 1982a).

Transport of spermatozoa has been improved by adding to semen or administering to females such compounds as prostaglandin $F_{2\alpha}$, oxytocin, oestradiol, phenylephrine or ergonovine. Oestradiol, prostaglandin $F_{2\alpha}$, phenylephrine and ergonovine administered to rabbits at insemination each increased fertilisation rates (Hawk, 1983).

The pattern of spermatozoa migration into and through the rat oviduct varies with the stage of the oestrous cycle, being dependent on oestradiol and progesterone. Exogenous oestradiol facilitated spermatozoa migration into the oviduct and progesterone antagonized this effect, whereas progesterone alone had no effect (Orihuela *et al*., 1999). In this study, concomitant treatment with oestradiol and progesterone induced adhesion of spermatozoa to the oviduct epithelium.

A local transfer of follicular hormones from the ovarian vein to the ovarian and utero-tubal arteries has been proposed as a means of regulating the function of the oviduct. Elevated concentrations of steroids and prostaglandins are present, thereby facilitating the periovulatory phase of sperm transport from the caudal portion of the oviduct to the site of fertilization (Hunter *et al*., 1983).

Superovulatory treatments in ewes have been shown to interfere with transport of spermatozoa. Evans and Amstrong (1984) examined the number of spermatozoa in the uterine horns and oviducts 24 hours after insemination in ewes superovulated with either pregnant mare serum gonadotrophin or follicle stimulating hormone. While pregnant mare serum gonadotrophin and follicle stimulating hormone resulted in an equivalent superovulatory response, there was a marked reduction in recovery of spermatozoa when compared with unstimulated animals, with the greatest reduction attributable to pregnant mare serum gonadotrophin treatment. It is believe that an abnormal hormonal milieu induced in the cervix and uterus by superovulation treatments, particularly pregnant mare serum gonadotrophin either interfered with the transport of spermatozoa or reduced the motility of spermatozoa.

The role of oxytocin in the transport of spermatozoa is unclear. Pulses of oxytocin have been measured in the peripheral blood of animals in oestrus including the ewe particularly when the male was present (Gilbert *et al.,* 1991; Nikolapoulus *et al*.,2000; Langendijk *et al*., 2003), and the amount of oxytocin receptor mRNA peaks in the uterus and cervix at oestrus (Stevenson *et al*., 1994; Fuchs *et al*., 1996). However, earlier studies showed that administration of oxytocin to ewes decreased the number of spermatozoa that reached the oviduct (Lighfoot and Restall, 1971).

Exogenous oxytocin will increase the number of contractions of the uterus and oviduct of the ewes (Gilbert *et al*., 1992) and while this might be significant in the rapid transport of spermatozoa to the oviducts, as noted previously, it is not this population of spermatozoa that fertilise oocytes. A more significant role of oxytocin could be the softening of cervical tissue though stimulation of prostaglandin E_2 synthesis (Fuchs *et al*., 1996) that in turn could make it easier for spermatozoa to pass into the cervix from the anterior vagina.

2.6 Distribution of Spermatozoa in the Female Reproductive Tract

The distribution of spermatozoa within the female reproductive tract after either natural mating or artificial insemination varies between and within animals. A summary of the published information on the distribution and motility of

spermatozoa in female reproductive tract of farm animals is provided in Table 2.2 and Table 2.3.

The number of spermatozoa recovered from the vagina and cervix of ewes decreases with collection time after insemination, but increased within the uterus and oviduct (Table 2.2a) (Quinlivan and Robinson, 1969; Mattner and Braden, 1969b). More spermatozoa were recovered from the reproductive tract of ewes (Table 2.2 a,b,c) at natural oestrus than those with synchronized oestrus (Quinlivan and Robinson, 1969; Allison and Robinson, 1970; Hawk and Conley, 1971; Allison and Robinson, 1972; Hawk and Cooper,1977). Mattner and Braden (1969b) recovered more spermatozoa from the reproductive tract of ewes after insemination at early oestrus than at late oestrus. Hawk and Cooper (1975) recovered more spermatozoa from the vagina of ewes inseminated at late oestrus than at early oestrus although the number of spermatozoa recovered from the cervix, uterus and oviduct was similar (Table 2.2 b).

In one experiment, Hawk and Cooper (1977) recovered 67 x 10^3 spermatozoa from the uterus and 100 from the oviducts two hours after natural mating but in another experiment (Hawk and Cooper, 1984), they recovered less spermatozoa from the oviducts (between 50 and 64) and the uterus (20 x 10^3) two hours after insemination (Table 2.2 c). The number of spermatozoa recovered from the oviducts is variable because different authors have used different insemination doses. The work of Quinlivan and Robinson (1969) showed that spermatozoa can be recovered from the oviducts of ewes one hour after mating and that the peak number are present at 24 hours (Table 2.2 a). In the pig, First *et al.* (1968) recovered 98 and 54 spermatozoa from the oviducts 15 minutes and 24 hours after insemination respectively (Table 2.2 b). The number of spermatozoa recovered from the ampulla of ewes and sows varied depending on the collection time after insemination (Schott and Phillips, 1941; Quinlivan and Robinson, 1969; Lane *et al*., 1993; Kunavongkrit *et al*., 2003).

Table 2.2 Distribution of spermatozoa in the female reproductive tract of farm animals.

(a)

Sample Collected *	Stage of Oestrous cycle	Vagina (x 10^3)	Cervix (x 10^3)	Uterus (x 10^3)	Oviduct			Number of sperm inseminated	Animal	References
					C	I	A			
1 h	O/us	12,300	1,510	10	-	160	50	500 x 10^6	Sheep	Quinlivan and Robinson, 1969
12 h	O/us	562	437	17	-	660	250			
24 h	O/us	646	741	62	-	3,800	650			
36 h	O/us	457	468	28	-	510	200			
48 h	O/us	65	107	12	-	160	170			
1 h	O/s	12,300	1,350	10	-	40	50			
12 h	O/s	22	562	8	-	270	100			
24 h	O/s	6	81	6	-	260	110			
36 h	O/s	4	31	6	-	240	210			
48 h	O/s	1	9	3	-	80	90			
4 h	O/early	-	17,200	101	13	-	-	550 x 10^6	Sheep	Mattner and Braden, 1969b
	O/late	-	530	1	2	-	-			
24 h	O/early	-	3,170	170	4,960	-	-			
	O/late	-	40	37	7	-	-			
24 h	O/us	-	-	-	2,500	-	-	400 x 10^6	Sheep	Allison and Robinson, 1970
	O/10 mg P	-	-	-	632	-	-			
	O/30 mg P	-	-	-	1,900	-	-			
	O/90 mg P	-	-	-	2,300	-	-			
	PMSG- O/500 IU	-	-	-	1,900	-	-			
12 h	O/early	-	-	-	-	1,233	-	100 x 10^6	Goat	Cox *et al*., 2002

* = after insemination, h = hours, O = oestrus, us = oestrus unsynchronised, s = oestrus synchronised, PMSG = pregnant mare serum gonadotrophin, P = progesterone, IU = international unit, C = complete oviduct, I = isthmus, A = ampulla, - = no data

Table 2.2 (Continued)

(b)

Sample Collected *	Stage of Oestrous cycle	Vagina (x 10^3)	Cervix (x 10^3)	Uterus (x 10^3)	Oviduct (x 10^3)			Number of sperm inseminated	Animal	References
					C	I	A			
24 h	O/us	2,400	240	10	2.1	-	-	25 x 10^6	Sheep	Hawk and Conley, 1971
	O/MPA	60	20	4.4	0.9	-	-			
2 h	O	123,000	30,800	23	-	-	-	500 x 10^6	Sheep	Allison, 1972
24 h	O	349	4,267	68	-	-	-			
24 h	O/us	20	16	2	-	-	-	500 x 10^6	Sheep	Allison and Robinson, 1972
	ODB- O/50 ug	20	18	5	-	-	-			
	O/ us	18	16	3	-	-	-			
	P- O/20 mg	20	18	4	-	-	-			
24 h	O-early	17,000	3,100	30	0.22	-	-	NM	Sheep	Hawk and Cooper, 1975
	O-late	50,000	3,000	10	0.27	-	-			
2 h	O/us	-	-	9,645	-	0	0	750 x 10^6	Sheep	Lane *et al*., 1993
22 h	O/us	-	-	30,458	-	2.445	150			
15 min	O	-	-	9,290	0.098	-	-	40 x 10^9	Pig	First *et al*., 1968
	Luteal	-	-	15,880	0.073	-	-			
24 h	O	-	-	400	0.054	-	-			
	Luteal	-	-	800.6	0.091	-	-			
12 h	O/s	58,400	3,400	1,700	30	-	-	1000 x 10^6	Cattle	Mitchell *et al*., 1985
	O/s	24,400	1,600	400	6	-	-	420 x 10^6	(uterine insemination)	

* = after insemination, h = hours, min = minutes, O = oestrus, us = oestrus unsynchronised, s = oestrus synchronised, PMSG = pregnant mare serum gonadotrophin, P = progesterone, IU = international unit, C = complete oviduct, I = isthmus, A = ampulla, - = no data, MPA = medroxy progesterone acetate, ODB = oestradiol benzoate, NM = natural mating

Table 2.2 (Continued)

(c)

Sample Collected *	Stage of Oestrous cycle	Posterior Cervix (x 10^3)	Mid-cervix (x 10^3)	Anterior Cervix (x 10^3)	Uterus (x 10^3)	Oviducts (x 10^3)	Number of sperm inseminated	Animal	References
0.5 h	O/early	8,000	4,600	120	-	-	800 x 10^6	Sheep	Mattner and Braden, 1969 a
4 h	O/early	4,000	3,500	1,900	-	-			
30 min	O	1,200	100	8	-	0.078	400 x 10^6 (frozen)	Sheep	Lightfoot and Salamon, 1970
	O	7,000	500	42	-	0.062	(fresh semen)		
2 h	O/us	29,000	18,000	2,100	67	0.1	NM	Sheep	Hawk and Cooper, 1977
	PGF2α - O/ s	12,000	3,000	179	15	0.1			
	MPA – O/ s	6,700	3,700	204	22	0.1			
4 h	O/ us	900	1,200	800	10	1.7	NM	Sheep	Hawk *et al*., 1978
22 h	O/ us	1,500	2,300	800	70	2.1			
2 h	O/ us	16,000	8,600	1,300	20	0.064	0.2 ml	Sheep	Hawk and Cooper, 1984
	O/ s	11,000	10,000	1,500	20	0.050			

* = after insemination, h = hours, min = minutes, O = oestrus, us = oestrus unsynchronised, s = oestrus synchronised, NM = natural mating, PGF2α = prostaglandin F2 alpha, MPA = medroxy progesterone acetate, - = no data

Table 2.2 (Continued)

(d)

Sample Collected *	Stage of Oestrous cycle	PU	AUR	AUL	UTJ	Oviduct C	Oviduct I	Oviduct A	Number of sperm inseminated	Animal	References
11 min	O	1	1	-	-	-	-	-	NM	Sheep	Schott and Phillips, 1941
14 min	O	1	1	-	-	1	-	-			
17 min	O	2	3	-	-	2	1	-			
20 min	O	1	2	-	-	1	-	18			
23 min	O	-	-	-	-	1	-	5			
26 min	O	-	1	-	-	-	-	8			
29 min	O	-	-	-	-	-	1	1			
32 min	O	-	1	-	-	-	-	4			
35 min	O	-	-	-	-	-	-	3			
40 min	O	-	-	-	-	-	-	1			
12 h	O	-	165,000	165,000	333,000	-	400	100	3,000 x 10^6	Pig	Langendijk *et al*., 2002
5-6 h	O/early	-	-	-	-	4	-	-	10,000 x 10^6	Pig	Kaeoket *et al.*, 2002
	O/late	-	-	-	-	3	-	-			

* = after insemination, PU = posterior uterus, AUR = anterior uterus-right, AUL = anterior uterus-left, UTJ = utero-tubal junction, C = complete oviduct, I = isthmus, A = ampulla, O = oestrus, NM = natural mating, min = minutes, - = no data

(e)

Sample Collected *	Stage of Oestrous cycle	Caudal uterus (x 10^3)	Cranial uterus (x 10^3)	Utero-tubal junction	Caudal isthmus	Cranial isthmus	Ampulla	Number of sperm inseminated	Animal	Reference
3 h	O/us	21,537	9,653	125,653	1,633	112	32	3 x 10^9	Pig	Kunavongkrit *et al.,*2003
12 h	O/us	201	548	105,365	2,409	39	18			

* = after insemination, h = hours, O = oestrus, us = oestrus unsynchronised

Table 2.3 The percentage of motile spermatozoa in the reproductive tract of farm animals.

Sample Collected *	Stage of oestrous cycle	cervix	Uterus	Oviducts	Number of sperm inseminated	Animal	References
24 h	O/early	71%	49%	45%	NM	Goat	Mattner, 1968
22 h	O/early	64%	52%	38%	NM	Cattle	
15 min	O		79%	60%	40 x 10^9	Pig	First *et al*.,1968
	Luteal		79%	35%			
24 h	O		0%	29%			
	Luteal		0%	30%			

* = after insemination, h = hours, min = minutes, O = oestrus, NM = natural mating

2.7 Retrograde Loss of Spermatozoa

Spermatozoa may be lost from the genital tract in three ways, (1) by voidance to the exterior from the vagina (Austin, 1957; Reid, 1965; Steverink *et al*., 1998), (2) by passage into the peritoneal cavity (Mattner and Braden, 1963) and (3) by phagocytosis within the tract (Reid, 1965; Pursel *et al*., 1978; Matthijs *et al*., 2000; 2003). Many of the spermatozoa in an inseminate whether deposited in the vagina, cervix or uterus of the cow drain from the female reproductive tract within a few minutes or hours after insemination, and remaining spermatozoa are removed from the tract by slower drainage or phagocytosis. In most cows over 60% of spermatozoa inseminated into the uterus are lost from the tract within 12 hours (Mitchell *et al*., 1985), and these findings were corroborated by Nelson *et al*. (1987). In ewes, the number of spermatozoa recovered from the cervix and vagina was greater at 2 hours than at 24 hours after insemination (Allison, 1972).

Gallagher and Senger (1989) reported that retention of spermatozoa by the cow reproductive tract did not differ when cornual insemination was compared to uterine body insemination, and spermatozoa movement into the vagina from the uterus was similar in both cases. When spermatozoa were deposited in the midcervix of the cow, spermatozoa loss was almost twice that after cornual deposition. The cumulative percentage of spermatozoa recovered from the vagina of heifers in an 8 hour collection period was similar for insemination into the uterine horns (17.9%)

and uterine body (18.5%) whereas that cumulative percentage of spermatozoa recovery from the vagina was greater for cervical deposition (59.1%) than for that into the uterine horns (30.9%) (Gallagher and Senger,1989).

Tilbrook and Pearce (1986) showed that many spermatozoa inseminated into the vagina of the ewe were lost by drainage through the vulva. The rate of loss was not affected by the motility of spermatozoa or oestrus state of the ewe. Spermatozoa were removed from the vagina during withdrawal of the penis after intromission and the extent of this loss varied between rams and with the volume of semen already in the vagina. Up to half of the inseminate was lost in this way when there was 0.5 ml of semen in the vagina, but only 11% was lost when the volume of inseminate was 0.1 ml.

Quinlivan and Robinson (1969) reported that most of 500 million spermatozoa deposited in the cervices of ewes had been lost within one hour, which suggests rapid loss by drainage. Moreover, there is evidence in the ewe that the retrograde loss of frozen-thawed spermatozoa is greater than fresh spermatozoa following insemination into the uterus (Gillan *et al.,* 1999).

2.8 The Post-insemination Inflammatory Response and Loss of Spermatozoa by Phagocytosis

Insemination induces a rapid and transient inflammatory reaction in the female reproductive tract. Soon after deposition of semen in the female reproductive tract, large numbers of neutrophils and macrophages accumulate within the superficial cervical mucosa and cervical lumen (Mattner, 1968; Pandja and Cohen, 1985) or within the endometrium and uterine lumen of species in which semen is deposited in the uterus (Kotilainen *et al*., 1994; Troedsson *et al*., 1998; Rozeboom *et al*., 1998, 1999). The inflammatory response to spermatozoa and seminal plasma appears to be a normal reaction after insemination and in addition to the likely mechanism to remove any microorganisms deposited with the inseminate, the phagocytic cells remove spermatozoa over a period of 24 to 36 hours after insemination. Placement of a large number of spermatozoa within the ligated uterine horn of the rabbit has

shown that only 20% of the spermatozoa can be recovered six hours later (Howe, 1967). Many of the spermatozoa were phagocytosed by neutrophils and macrophages. Evidence is accumulating based on studies mainly in mice that the post-insemination inflammatory reaction produces a range of cytokines that seem to promote the development of the embryo and implantation as well as inducing maternal immune tolerance to the conceptus (Robertson *et al*., 1997; Robertson and Sharley, 2001).

2.9 Interaction of Spermatozoa with Oviduct Epithelium

It is now widely accepted that the oviduct is not simply a passive tube supporting the union of spermatozoa and oocytes. The oviduct is regarded as containing a complex milieu that significantly influences the migration and function of spermatozoa as well as oocytes and cleavage-stage embryos. A number of studies both *in vivo* and *in vitro* have shown that the oviductal environment extends the fertile life of spermatozoa, a feature that is particularly important in animals such as ruminants which ovulate after the end of oestrus. The function of the isthmus is to store spermatozoa, a feature that has been demonstrated in many animals including sheep (Hunter and Nichol, 1983), cattle (Hunter and Wilmut, 1984), pigs (Hunter, 1984), rabbits (Overstreet and Cooper, 1978), hamsters (Smith *et al*., 1987), mice (Suarez, 1987) and rats (Shalgi and Kraiser, 1978). As discussed previously (see Section 2.6), in most mammalian species very few spermatozoa are found in the oviduct, including sheep (Schott and Phillips, 1941; Quinlivan and Robinson, 1969; Mattner and Braden, 1969b; Alison and Robinson, 1970; Lightfoot and Salamon, 1970; Hawk and Conley, 1971; Allison, 1972; Hawk and Cooper, 1975; Hawk and Cooper, 1977; Hawk *et al*., 1978; Hawk and Cooper, 1984; Lane, 1993), goats (Cox *et al*., 2002), cattle (Mitchell *et al*., 1985), and pigs (First *et al*., 1968; Kaeoket *et al*., 2002; Langendijk *et al*., 2002).

Storage of spermatozoa within the isthmus involves intimate contact between spermatozoa and the epithelial cells of the isthmus. In addition to this direct contact, spermatozoa are also bathed in a variety of secretions derived from of oviductal epithelium (Leese *et al.,* 2001). One group of secretory products which have

received considerable attention is the family of glycoproteins identified as oestrus-associated glycoprotein (reviewed by Buhi, 2002).

After a period of storage in the isthmus some spermatozoa will detach, acquire hyperactivity and move into the ampulla. It is not clear what determines which spermatozoa detach and if there is a definite period during which they have to remain attached in order to be competent to fertilise an oocyte although data from work on several different species indicates that uncapacitated spermatozoa remain attached to oviductal epithelium (Thomas *et al*., 1995; Levebre and Suarez, 1996; Fazeli *et al.,* 1999). Evidence is accumulating that the adherence to the isthmus epithelium may be acting as a mechanism to select those spermatozoa that are undamaged or have superior fertilizing ability (Gualtieri and Talevi, 2003). There is evidence to support the view that changes during capacitation play a key role in the release of spermatozoa and that sulfated glycoconjugates are modulators of adhesion and release of spermatozoa (Smith and Yanagimachi, 1991; Gualtieri and Talevi, 2000; Talevi and Gualtieri, 2001). From a biological point of view it would make sense that the mechanism of ovulation or some other immediate consequence of ovulation such as changes in hormone concentrations in the isthmus could be the controller of detachment of spermatozoa.

2.10 Capacitation

When mammalian spermatozoa are released from the male reproductive tract at ejaculation or are recovered by micropuncture of the cauda epididymidis they are morphologically complete and capable of motility but are not able to fertilize. On the other hand, spermatozoa recovered from the uterus or oviduct several hours after insemination or after *in vitro* culture in a suitable physiological medium have the capacity to fertilise. This phenomenon of the capacity to fertilise is called capacitation and was first described independently by Austin (1951) and Chang (1951). Capacitation has been the subject of numerous investigations by many research workers since the original observation by Austin and Chang. The time taken for spermatozoa to become functional after exposure to permissive conditions is species-dependent eg.1-1.5 hours in sheep and 5-6 hours in rabbits (Hunter, 1988).

Broadly speaking, the process of capacitation involves the removal from the spermatozoal surface of a coating of glycoprotein molecules acquired during the sojourn of spermatozoa in the epididymis with subsequent changes to molecular organization of the spermatozoal cell membrane. However, there are no visible changes to the morphology of spermatozoa and the exact molecular mechanisms involved in capacitation are not fully understood. The determination of when spermatozoa have undergone capacitation requires the use of various staining techniques (Kaya *et al*., 2001; Rijsselaere *et al*., 2002; Somfai *et al.*, 2002).

The capacitation status of spermatozoa in a variely of species can be determined by the chlortetracycline-assay (Perez *et al*., 1995; Mattioli *et al*., 1996; Gillan *et al*., 1997; Kaul *et al*., 2001; Rathi *et al*., 2001). This assay distinguishes three stages of sperm activation: non-capacitated, capacitated acrosome-intact and capacitated acrosome-reacted. An assay using the flow cytometric technique with stains such as merocyanine 540 have been recently investigated (Rathi *et al.*, 2001) and other assays have been used to detect the acrosome reaction (Kitiyanant *et al*., 2002).

2.10.1 *In vivo* capacitation and the acrosome reaction

Considering the importance of capacitation, there is surprisingly little detailed information on the processes *in vivo*. The information is mainly confined to work conducted on rabbits and humans and there is scant information on other species.

Based on studies in humans, capacitation may start while spermatozoa are passing through the cervix (Gould *et al*., 1985; Lambert *et al*., 1985) but what is not known is whether all spermatozoa start the process in the cervix. Studies with rabbits (Bedford, 1969) indicated that spermatozoa can complete capacitation in the uterus but it is not clear if these spermatozoa commenced capacitation in the cervix. It also seems that the rabbit oviduct has a greater innate capacitation potential as rabbit spermatozoa exposed to the uterus and the oviduct will complete capacitation faster (within six hours) than those only exposed to the uterus (15 hours) (Bedford, 1969; Bedford, 1970).

Biologically, it would make sense for the fertilising population of spermatozoa to only commence capacitation in the anterior uterus so that they could complete the process in the isthmus and be prepared to leave the isthmus at the appropriate time to move to the ampulla and then undergo the acrosome reaction. It is likely that those spermatozoa that undergo a substantial part of the capacitation process in the cervix and posterior uterus fail to fertilise. If this is the case, then the question is what determines which spermatozoa undergo capacitation at different sites in the reproductive tract.

Albumin is the major protein in the female reproductive tract secretion and is an important component during *in vivo* capacitation. Albumin is believed to facilitate the efflux of sterols (mainly cholesterol) from capacitating spermatozoa (Dow and Bavister, 1989). The loss of cholesterol has been suggested to affect the sperm plasma membrane lipid bilayer and make it "fusogenic" (for review see Cross, 1998).

Once spermatozoa have undergone capacitation they also change in other respects. They can respond to a chemotactic signal from the ovulated oocyte (Eisenbach, 1999) and have a greater ability at least in the bull to bind to the zona pellucida of oocytes (Topper *et al*., 1999).

The other feature that capacitated spermatozoa have is that they can undergo the acrosome reaction, an exocytotic process that allows spermatozoa to bind to and then penetrate the zona pellucida. The loss of cholesterol during capacitation promotes membrane priming and membrane fusion. The net result of membrane fusion is the release of acrosomal contents (glycohydrolases, proteases, etc) at the site of sperm-oocyte binding (Tulsiani *et al*., 1998; Abou-Haila and Tulsiani, 2000). After penetration has been achieved, acrosome-reacted spermatozoa are capable of fusing with the oocyte plasma membrane, thereby activating the oocyte and setting in train the completion of the second meiotic division and the initiation of the metabolic events that will sustain early embryonic development (see review by Talbot *et al*., 2003).

2.10.2 *In vitro* capacitation

Capacitation is possible without any contribution from the secretions of the female reproductive tract and can be induced *in vitro* by incubation in many media that over the years have been especially developed to support *in vitro* fertilisation. The component of these media that is responsible for induction of capacitation is bicarbonate / CO_2 (Suzuki *et al*., 1994; Shi and Roldan, 1995). The changes in spermatozoa that are induced by bicarbonate / CO_2 include changes to the surface coating, changes in the behaviour of lipids of the plasma membrane and increased affinity to bind to the zona pellucida (Ashworth *et al*., 1995; Harkema *et al.,* 1998; Gadella and Harrison*,* 2002).

There are numerous reports in the literature that describe the various biochemical events that occur within a spermatozoon during the process of capacitation as well as the acrosome reaction. Example of reviews of the topic are by Yanagimachi (1989), Harrison (1996) and Breitbart and Naor (1999).

A variety of substances when added to culture media also help to promote the capacitation process. The presence of bovine serum albumin seems to be necessary for bicarbonate- induced capacitation at least in equine spermatozoa (Colenbrander *et al*., 2002). Heparin and micromolar concentrations of caffeine promote capacitation (Cosconi *et al*., 2001; O'Flaherty *et al.*, 2002) whereas seminal plasma will inhibit or delay the capacitation process (Suzuki *et al*., 2002). It has also been observed that the membrane changes that occur after cryopreservation and thawing of spermatozoa are functionally equivalent to capacitation (Gillan *et al*., 1999; Parker *et al.,* 2000; Cormier and Bailey, 2003). Current evidence indicates that the mechanism controlling the intracellular Ca^{2+} concentration plays a pivotal role in controlling the capacitation process (Fraser *et al*., 1995; Arnoult *et al*., 1999).

An example of the time-frame over which spermatozoa in a fresh ejaculate will undergo capacitation *in vitro* is the work reported by Huo *et al.* (2002). They incubated boar semen at 39 ^{o}C and found that 3%, 7%, 9%, 15%, 20% and 33% were capacitated after 0, 1, 2, 4, 8 and 12 hours incubation respectively. By 24 hours there

were no live capacitated spermatozoa. The capacitation process was considerably delayed if the semen was stored at 15 ^{o}C or 20 ^{o}C.

2.11 Morphological and Motility Characteristics of Spermatozoa

The mammalian spermatozoon has two main components, the head and the flagellum or tail, which are joined at the neck. The other regions of the flagellum are the middle piece, the principal piece and the end piece (Eddy and O'Brien, 1994). The flagellum provides the motile force necessary for spermatozoa to reach the surface of the oocyte and achieve fertilisation. The length of a spermatozoon varies between species. In humans, it is 60 μm and the flagellum is 55 μm (Baccetti, 1984). The rabbit spermatozoon is 46 μm in length, mice 120 μm, rats 140 μm and Chinese hamster 250 μm (Phillips, 1972), whereas the bovine spermatozoon is 53.6 μm, goat 59.4 μm, pig 54.6 μm and the ram spermatozoon is 67.2 μm with a head length of 8.2 μm (Cummins and Woodall, 1985).

Computer-assisted sperm morphology analysis has been used to determine the detailed dimensions of the head of spermatozoa. Gravance *et al*.(1998) reported the following data for ram spermatozoa: length, 8.08 μm; width, 4.80 μm; width-length ratio 0.59 with an area of 29.13 μm^2 and a perimeter of 23.93 μm. Sancho *et al.* (1998) reported that there were significant differences in ram sperm dimensions between the four fixation techniques used. The mean morphometric measurements for the spermatozoal head of bulls were area 27.3 μm^2, perimeter 25.36 μm, length 8.65 μm, width 4.4 μm and width-length ratio of 0.5 (Gravance *et al.,* 1996).

The evaluation of motility and morphology of spermatozoa is an essential component of the examination of semen quality and in the establishment of the correlation between semen quality and fertility. Traditionally, assessment of the quality of spermatozoa has been based on subjective evaluation of parameters such as mass and individual motility, and objective parameters such as sperm concentration and morphology abnormalities. In recent years, computer-aided semen analysis (CASA) systems have been developed that allow an objective assessment of different cell characteristics such as motion, velocity and morphology (Liu *et al*.,

1991; Krause, 1996; Zinaman *et al*., 1996; Farrel *et al*., 1998; Steigerwald and Krause, 1998; Cotzee *et al*., 1999; Iguer-Ouada and Verstegen, 2001). Computer-assisted semen analysis was first proposed by Dott and Foster (1979) and is now used in many laboratories for the definition and investigation of infertility in men and animals as well as for research studies. However, CASA results in humans are unreliable at spermatozoa counts of less than 20 million/ml and post-thawed motility is generally underestimated (Sindhu *et al*., 1998).

Movement of a spermatozoon can be classified in three patterns: a regular trajectory, curved trajectory and high-amplitude trajectory (Mortimer, 2000). The kinematic values determined for each spermatozoon cover the velocity of movement, the width of the sperm head's trajectory, and frequency of the change in direction of the sperm head (David *et al.,* 1981). The velocity values are curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP). The value of VCL is always the highest, VSL is lowest and VAP in between (Mortimer, 2000). Consensus on operating procedures for the use of CASA as well as definition of terminology has now been achieved (Davis and Siemers, 1995; Mortimer, 2000). Table 2.4 lists the commonly used definitions and these are partially illustrated in Figure 2.1.

Table 2.4 Definitions of sperm kinematics measures (Davis and Siemers, 1995).

Symbol	Name	Definition	Units
VAP	Average path velocity	Time-average velocity of the sperm head along its average trajectory	μm/s
VSL	Straight-line velocity	Time-average velocity of the sperm head along a straight line from its position to its last position	μm/s
VCL	Curvilinear velocity	Time-average velocity of the sperm head along its actual trajectory	μm/s
STR	Straightness	Straightness of the average path (VSL/VAP)	%
ALH	Amplitude of lateral head displacement	Amplitude of variations of the actual sperm head trajectory about its average trajectory (the average trajectory is computed using a rectangular running average)	μm
BCF	Beat cross frequency	Time-average rate at which the actual sperm trajectory crosses the average path trajectory	Hz
LIN	Linearity	Linearity of the curvilinear trajectory (VSL/VCL)	%

2.12 Hyperactivation of Spermatozoa

Hyperactivated motility is the vigorous and high amplitude beating of the flagella of spermatozoa that is usually seen in spermatozoa retrived from the oviductal ampulla. The topic has been recently reviewed by Ho and Suarez (2001b). While studying hamster spermatozoa *in vitro*, Yanagimachi (1969) observed that spermatozoa began beating their flagella much more vigorously when they acquired the ability to fertilise oocytes. This was the first observation of hyperactive movement of spermatozoa and Yanagimachi proposed that vigorous beating of the flagella facilitated the penetration of the zona pellucida by spermatozoa. Subsequent *in vivo* observations showed that hyperactivated spermatozoa were present in the oviducts of sheep (Cummins, 1982) and mice (DeMott and Suarez, 1992). DeMott and Suarez (1992) noted that only spermatozoa showing hyperactivity were able to move away from the oviductal epithelium.

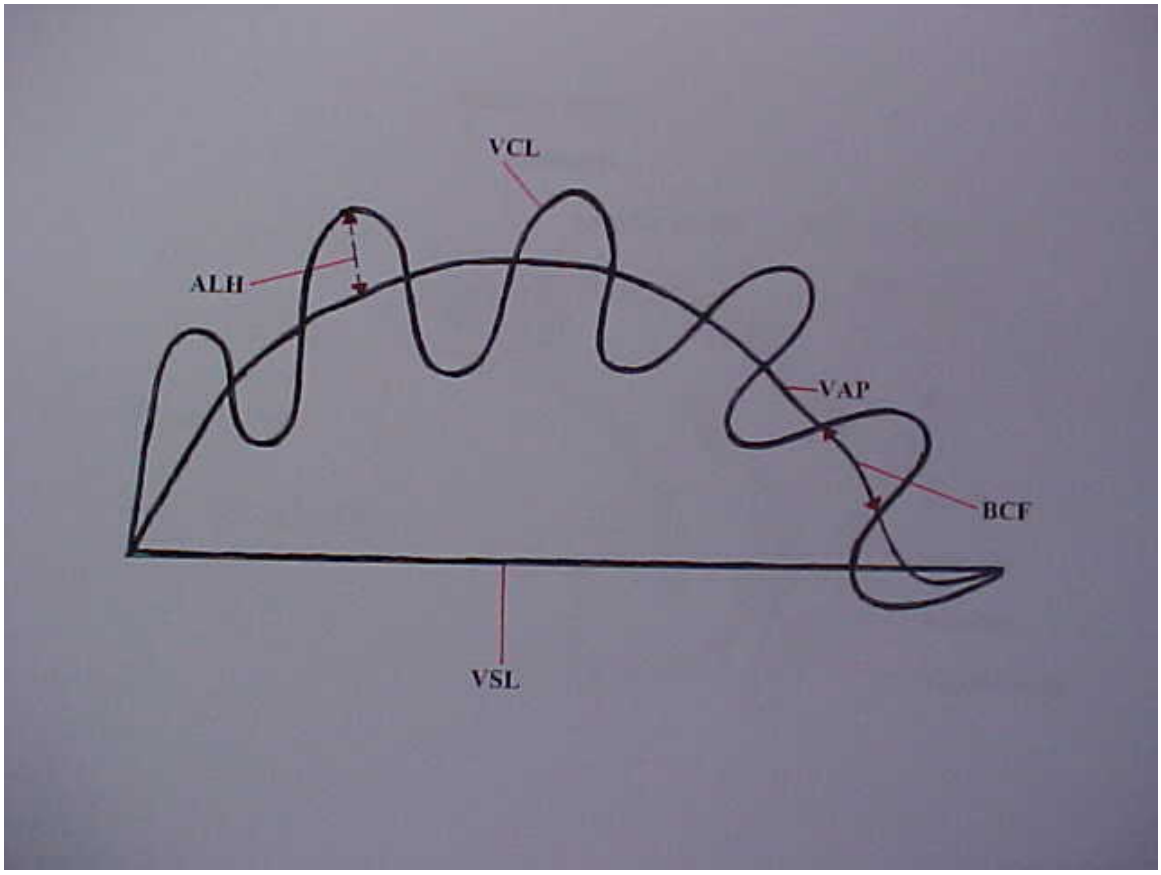


Figure 2.1 Schematic diagram of spermatozoon kinematic measures by CASA (modified from Davis and Siemers, 1995).
VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

The flagella of hyperactivated spermatozoa are thrown into deeper bends and their beating is usually less symmetrical than that of normally activated spermatozoa. As a result, the spermatozoa tend to swim vigorously in circles on a microscope slide (Ho and Suarez, 2001a). In addition and depending on the species, the spermatozoa roll rapidly as they swim.

Ram spermatozoa will exhibit hyperactivated motility under *in vitro* capacitation conditions (Mortimer and Maxwell, 1999). Hyperactivated spermatozoa had a significantly higher flagellar beat angle, beat envelope and significantly lower flagellar curvature ratio values than non-hyperactivated spermatozoa. These criteria were refined by consideration of 60 hertz computer-assisted semen analysis-derived trajectories, and ram sperm hyperactivation was defined by: curvilinear velocity more than 250.0 μm/s and straight-line velocity less than or equal to 100.0 μm/s and linearity less than or equal to 30% and amplitude of lateral head displacement maximum more than or equal to 9.0 μm (Mortimer and Maxwell, 1999).

Hyperactivated spermatozoa of Golden hamsters had remarkably asymetrical flagella waves of large amplitude because either the bends in the same direction as the hook of the head (referred as the “pro-hook bend”) or the bends in the opposite direction to the hook of the head (referred as the “anti-hook bend”) markedly increased their curvature. Acrosome-reacted spermatozoa had symmetrical flagellar waves of large amplitude because both the pro- and anti-hook bend remarkably increased their curvature. Beat frequency significantly decreased while the wavelength of flagellar waves increased after hyperactivation and the acrosome reaction (Ishijima *et al*., 2002).

Many studies have been conducted on the likely physiological signals and biochemical mechanism that operate in hyperactivation. A number of the components of the oviductal fluid including Ca^{2+}, cAMP, bicarbonate and metabolic substrates seem to be essential for the initiation and maintenance of hyperactivation (reviewed by Ho and Suarez, 2001b).

It makes biological sense for spermatozoa to develop a high degree of motility just prior to ovulation. Once spermatozoa detach from the isthmus epithelium they still

need to negotiate fluid and mucus in the oviduct as well as penetrating the cumulus mass in order to fertilise the oocyte. In many respects this is a difficult journey and spermatozoa need considerable propulsive forces to penetrate these barriers.

2.13 Summary and Conclusion

The experimental evidence reviewed in this Chapter shows that after deposition of semen in the female reproductive tract either by natural or artificial means, numerous physiological and biochemical changes occur which influence the life of spermatozoa and determine whether they reach the ultimate goal of fertilization of the oocyte. While the scientific literature has described a number of the features of the life of spermatozoa in the reproductive tract, relatively little is known, except for perhaps the role of the isthmus, of the detailed cellular communication between the female reproductive tract and spermatozoa.

A fundamental question is whether it is purely by chance that a small population of spermatozoa reaches the site of fertilisation or whether the process involves an active selection by the female reproductive tract of those spermatozoa most likely to achieve fertilisation. The literature has not addressed this issue to any extent but it is an issue of importance for the advancement of the efficiency of animal production.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

3.1.1 Rams

Nine Merino rams (eartag numbers R1, R3, R5, R6, R9, R10, R12, R13, R16) of proven fertility were available for use in these studies. These animals were grazed on irrigated natural pastures in a small internal paddock within the large animal facilities of the Biomedical and Tropical Veterinary Science precinct, Douglas campus, James Cook University and received supplementary feeding with a pelleted protein and energy supplement (400 g per ram per day) (Barastoc Graze-R 30; Ridley Agri Products Pty Ltd, Toowoomba, Queensland). The rams had been vaccinated against clostridial diseases (Ultravac 5 in 1; CSL, Parkville, Victoria) and were treated at intervals of three to six weeks for gastro-intestinal parasites (Promec-RV; Merial Australia Pty Ltd, Parramatta, New South Wales).

3.1.2 Ewes

Adult Merino ewes that had at least one lamb were used. The ewes were purchased as required from a grazier in the Hughenden district of North Queensland. Ewes were grazed on natural pastures in paddocks in the Biomedical and Tropical Veterinary Science precinct and were housed each night seven days a week in dog-proof pens. The ewes had been vaccinated against clostridial diseases, were regularly treated at intervals of three to six weeks for gastro-intestinal parasites, and had *ad libitum* access to a protein and mineral supplement (Weangro; Coleman Stock Feeds, Charter Towers, Queensland) from May to December.

3.1.3 Wethers

Two adult Merino wethers were used in these studies to detect oestrus. They were androgenised by subcutaneous injection of 350 mg testosterone propionate (Tepro-hormone; Virbac Australia Pty Ltd, Peakhurst, New South Wales) at 18 day intervals. The husbandry practices for wethers were the same as for the ewes.

3.2 Ethics Approval

Ethics approval to conduct studies on animals was given by James Cook University Animal Ethics Sub-Committee (approval number A 689).

3.3 Preparation of Media

Tyrode's albumin-lactate-pyruvate (TALP) and Hepes synthetic oviduct fluid (HSOF) media were used for dilution of ram semen and for the collection of mucus samples from the reproductive tract of ewes. The composition of these media is listed in Table 3.1.

3.4 Collection of Ram Semen

Semen was collected from adult rams by electroejaculation using standard procedures (Evans and Maxwell, 1987). The ram was manually restrained on its side within a building out of direct sunlight and the penis extruded. The penis was kept extruded by placing a piece of cotton gauze posterior to the glans penis to hold the extended penis and to direct the glans into a 15 ml sterile plastic centrifuge tube (Rohre/tube; Sarstedt, Germany). The collection tubes were kept in a polystryene box at about 39 ^{o}C. Electroejaculation was achieved by stimulation of the internal male accessory glands and nerves to the penis with a rectal probe connected to the mobile electrical stimulator (Electrojec; Ratex Instruments, Mitcham, Victoria). The electrical stimuli were given in a three seconds on and three seconds off pattern, with a gradual increase in voltage from zero volts to the optimum desired peak (five volts)

then returning to zero volts. An electroejaculation attempt was terminated if semen was not obtained after 16 stimulations. Semen was collected no more than twice from a particular ram within a 7 day period. At the completion of a semen collection, a small amount of antiseptic cream was applied to the glans penis before allowing the penis to retract into the prepuce. The prepuce and penis was gently massaged for about one minute to reduce any swelling that may have developed and to reduce any discomfort the ram may have experienced.

Table 3.1 Composition of modified Tyrode's albumin-lactate-pyruvate medium (TALP) and Hepes-synthetic oviduct fluid (HSOF) medium.

Constituents	Concentration (mg/ 100 ml)		Supplier
	TALP*	HSOF**	
NaCl	100 mM (584.4)	1007.7 mM (629.4)	Sigma, USA
KCl	3.1 mM (23.11)	7.16 mM (53.4)	BDH Chemicals, Australia
$CaCl_2$	2 mM (29.4)	1.17 mM (12.99)	Ajax Chemicals, Australia
$MgCl_2$	0.4 mM (3.81)	0.49 mM (4.7)	Sigma,USA
$NaHCO_3$	25 mM (210.1)	25.07 mM (210.6)	Ajax Chemicals, Australia
$NaH_2PO_4.2H_2O$	0.3 mM (4.68)	-	BDH Chemicals, Australia
L-Lactic acid	21.6 mM (242.14)	3.3 mM (36)	Sigma, USA
HEPES	10 mM (238.3)	15 mM (357.45)	Sigma, USA
Sodium pyruvate	1 mM (11)	0.33 mM (3.63)	Sigma, USA
BSA-V	6 mg/ml (600)	3.2 mg/ml (320)	Sigma, USA
KH_2PO_4	-	1.19 mM (16.2)	Ajax Chemicals, Australia
D-Glucose	-	4 mM (81)	Ajax Chemicals, Australia
Penicillin-G	-	75 µg /ml (7.5)	Sigma, USA
Streptomycin	-	50 µg/ml (5)	Sigma, USA
Kanamycin monosulfate	-	120 µg/ml (12)	Sigma, USA
Pyruvic acid	-	60 µg/ml (6)	Sigma, USA
Distilled water	100 ml	100ml	

* Modified from Parrish *et al*., 1988
** Modified from Tervit *et al*., 1972

3.5 Semen Analysis and Estimation of Spermatozoa Concentration

The volume of semen collected was recorded and the colour/consistency scored using the following macroscopic criteria: 0 = clear, 1 = cloudy, 2 = milky, 3 = thin creamy, 4 = creamy, 5 = thick creamy (Evans and Maxwell, 1987).

An aliquot of the semen sample (1 µl) was added to 199 µl of 9% NaCl in a test tube. After thorough mixing, this suspension was used to fill both chambers of a haemocytometer. A coverslip was placed on the sample and allowed to stand for one minute to allow the spermatozoa to settle. It was then placed on a microscope (Olympus BHA, Japan) and examined at 10 x magnification. Spermatozoa contained within five large squares (the four corners and center square) of each large chamber were counted. The concentration of spermatozoa in the semen sample was calculated by multiplying the mean of both counts x 10^7/ ml.

3.6 Determination of Motility and Velocity Parameters of Spermatozoa in Ram Semen

Fresh semen (50 µl) from a ram was diluted in either 600 µl of the TALP and HSOF medium. The diluted semen samples were held at 39 ^{o}C on a microscope warm stage (LEC Instruments, Scoresby, Victoria). This temperature was selected in order to approximate the temperature of the reproductive tract of the ewe. After three minutes, a sample was collected and the motility, velocity and head parameters of spermatozoa determined with a computer-aided semen analyser (CASA) (Hamilton Thorne Research-IVOS version 10, Beverly MA, USA).

3.6.1 CASA settings and definitions

In this study, the main software setting of the CASA were set as follows: 30 frames acquired per sec; frame rate, 50 Hz; minimum contrast, 20 %; minimum cell size 5 pixels; minimum static contrast, 30 %; straightness, 60%; low VAP cut-off, 5 µm/s; medium VAP cut-off, 25 µm/s; low VSL cut-off, 5 µm/s; head size non-motile, 15 pixels; head intensity, 90 pixels; static head size, limits 0.25-3.5 pixels; static head

intensity limits 0.1-2.5 pixels; static elongation limits 0-100 %. The temperature of the slide chamber was set at 39^{o}C.

The following are the definitions used for various motility and morphological features of spermatozoa.

- Motile is the population of spermatozoa that were moving at or above a minimum speed as determined by values defined under the setup of the CASA whereas the motility is the ratio of motile to non-motile spermatozoa.
- Progressive motility is the number of spermatozoa moving with both an average path velocity (VAP) > medium VAP cut off (MVV) and straightness (STR) > the threshold straightness (So).
- Rapid spermatozoa is the percentage of motile spermatozoa with VAP > MVV.
- Average path velocity (VAP) is the average spermatozoa path velocity of a spermatozoon in μm/s of the selected track.
- Straight-line velocity (VSL) is the straight line velocity (beginning of track to end of track) in μm/s of the selected track.
- Curvilinear velocity (VCL) is the point-to point track velocity (actual path) in μm/s of the selected track.
- Amplitude of lateral head displacement (ALH) is the mean width of the spermatozoal head of the oscillation, in μm.
- Beat cross frequency (BCF) is the frequency (in Hertz) with which the spermatozoal track crosses the spermatozoal path for the selected track.
- Straightness (STR) is the ratio of VSL/VAP, expressed as a percent, for the selecte track.
- Linearity (LIN) is the ratio of VSL/VCL, expressed as a percent, for the selected track.
- Elongation is the ratio of the head width to head length, expressed as a percentage, for the selected spermatozoa.
- Head area is the calculated head area of the selected spermatozoon in μm^2.

The overall population of spermatozoa was subdivided into four motility categories: rapid where VAP > MVV; medium where LVV (low VAP cut-off) < VAP < MVV; slow where VAP < LVV or VSL < low VSL cut off (LVS); and static, the fraction of all spermatozoa that were not moving during the analysis (Yeung *et al*., 1997).

3.7 Oestrus Synchronization and Mating

The time of oestrus in ewes was controlled by the intravaginal insertion of progesterone containing devices (Eazi-Breed CIDR; Pharmacia and Upjohn Pty Ltd, Rydalmere, New South Wales) for 13 days and then removed. Oestrus was expected to commence 36-48 hours later. Oestrus was controlled in groups of four ewes at a time. Twelve hours before expected oestrus, two ewes were placed with an androgenised wether wearing a marker harness and crayon (Donaghys Ram Crayon; Donaghys Industries Ltd, Christchurch, New Zealand) and an apron to prevent any possible intromission of seminal fluid (Figure 3.1). The apron was attached so that it would hang just anterior to the prepuce. Ewes in oestrus at 4.00 pm to 6.00 pm were mated the next morning (8.00 am to 9.00 am) whereas ewes in oestrus at 7.00 am to 9.00 am were immediately mated for one hour. A ewe was placed in a pen with a ram for one hour and the number of matings recorded. Throughout this thesis reference to a time period after mating refers to the number of hours after the end of the one hour mating period.

3.8 Collection of Samples from the Reproductive Tract of Ewes

Samples of fluid and mucus from various sites in the reproductive tract of ewes were collected into TALP medium for determination of motility parameters of spermatozoa and into HSOF medium for determination of capacitation status. Samples were collected into warm medium (39^{o}C) which had been dispensed (600 µl) into eppendorf tubes and kept warm in a polystryene box.

In initial experiments to study the motility of spermatozoa in the vagina, samples of vaginal mucus (200 – 400 µl) were collected from the anterior vagina at 1, 3, 6 and 24 hours after mating. The ewe was restrained and the sample collected by aspirating the vaginal mucus with a syringe attached to a plastic insemination pipette with visualisation being provided by a light source and vaginal speculum.

Figure 3.1 Photographs to illustrate an androgenised wether wearing a marker harness (white arrow) and apron (black arrow) used in the detection of oestrus in ewes. The ewe in figure A is not in oestrus and the ewe in figure B is in oestrus.

As preliminary studies showed that surgical collection of samples from the cervix, uterus and oviducts was not satisfactory, it was necessary to kill ewes to obtain samples. Ewes were euthanized at either 3, 6 or 24 hours after mating by an intra-

venous injection of concentrated barbiturate solution (Lethabarb; Virbac Australia Pty Ltd, Peakhurst, New South Wales). The reproductive tract was removed and the presence and diameter of the preovulatory follicle or the corpus haemorrhagica recorded. The oviducts were removed at the utero-tubal junction, put into plastic Petri dishes and placed in a polystryene box at 39^{o}C.

The vagina was opened and about 200 µl of vaginal mucus collected from the anterior vagina with a 1 ml tuberculin syringe. The posterior third of the cervix was opened, 200 µl of TALP medium added to the mucosa and the medium and mucus collected by aspiration with a 1 ml tuberculin syringe. The aspiration process was applied three times to the cervical mucosa, a technique that was used repeatedly in the collection of samples from the reproductive tract unless otherwise stated. The procedure was repeated with 200 µl HSOF medium.

Samples were collected from the mid and posterior thirds of the cervix, uterine body and mid-uterine horns. For the latter, a 2.5 cm section was opened midway between the uterine body and utero-tubal junction. The anterior 2.5 cm section of the uterine horn was then removed and 200 µl TALP flushed through the piece of uterine horn followed by 200 µl HSOF medium. The piece of uterine horn was then opened, pinned to a wax board, 100 µl of TALP medium carefully added to the mucosa and the fluid collected by aspiration with a tuberculin syringe. The process was then repeated with 100 µl HSOF medium. The sequence of collection of samples from the anterior vagina to anterior uterine horn was the same in all ewes. The collection process took about 20 minutes.

The isthmus and ampulla were separated from the remainder of the oviduct (each section was 4.5 cm in length). Six hundred µl of TALP medium was flushed through the isthmus using a 23 G needle followed by 600 µl of HSOF medium. The process was then repeated for the ampulla and then the other oviduct. The collection of oviduct samples was completed about 45 minutes after the commencement of collection of samples from the vagina.

3.8.1 Description of the procedures for analysis of the samples

One hour before the samples was analysed, the CASA (Hamilton Thorne Research-IVOS version 10; Beverley MA, USA) was turned on and the temperature of the analysis chamber set at 39 ^{o}C. Analysis chambers were kept at a temperature of 39 ^{o}C on a microscope warm stage (LEC Instruments, Scoresby, Victoria). Samples were kept in an insulated container containing warm water in bottles at 37 to 39 ^{o}C.

Samples in TALP medium were used to determine the motility, velocity and head characteristics of spermatozoa whereas the samples in HSOF medium were used to determine the capacitation status. Furthermore, eosin-negrosin staining was also used on the samples in TALP medium. The remaining samples in TALP and HSOF medium were used to determine the recovery of spermatozoa.

3.8.2 Determination of motility characteristics of spermatozoa in CASA

Two µl of each of the samples from the anterior vagina, posterior cervix, midcervix and anterior cervix in TALP medium were collected with a micropipette and then placed into a 20 micron analysis chamber (Standard Count; Leja, Nieuw-Vennep, The Netherlands). The chamber was inserted into the CASA and 100 to 200 spermatozoa were evaluated for motility, velocity and head morphology characteristics.

After thorough mixing of a sample, about half of the sample volume from the uterus and oviducts in TALP medium were filtered using a filterwell (Millicell-PC Culture Plate Insert; Millipore, Malshein, France). The filterwell was placed on tissue paper on a warm plate (39 ^{o}C) (Easy Veterinary Equipment, Ermington, New South Wales) until the volume was reduced to about 50 µl. Two µl of the sample was collected with a micropipette and placed into a 20 micron analysis chamber. The chamber was inserted into the CASA and about 100 spermatozoa were evaluated for motility, velocity and spermatozoa head morphology characteristics.

3.8.3 Preparation of samples for capacitation assay

Fifty µl of the sample in the HSOF medium was used to determine the capacitation status of spermatozoa in the female reproductive tract of ewes (see Section 3.9).

3.8.4 Determination of number of spermatozoa in samples

The number of spermatozoa recovered from each site in the reproductive tract was determined the next day. The number of spermatozoa in the remainder of the samples in TALP and HSOF medium from the anterior vagina was calculated using a haemocytometer (see Section 3.5). The volume of the remaining cervical and uterus samples was reduced to 100 µl, and after thorough mixing, 10 µl was placed on to a microscope slide. A coverslip (24 x 40 mm) was placed on the sample and allowed to stand for one minute to allow the spermatozoa to settle. The number of spermatozoa was counted by scanning the area under the coverslip and the total recovery of spermatozoa determined by multiplying the spermatozoa count by 10. The remainder of the samples from the oviduct in TALP and HSOF medium was reduced to 50 µl and after thorough mixing, 10 µl was placed onto a microscope slide. A coverslip was placed on the sample and allowed to stand for one minute to allow the spermatozoa to settle. Spermatozoa were counted in the area under the coverslip. The procedure was repeated a further four times until spermatozoa in all of the volume of 50 µl had been counted.

3.8.5 Measurement of head and tail length of spermatozoa

Spermatozoa were stained with nigrosin-eosin (Barth and Oko, 1989), the tail length and width, and length of the head were measured using an Olympus Eyepiece Micrometer on an Olympus microscope at 40 x magnification.

3.9 Chlortetracycline assay for Capacitation

The chlortetracycline (CTC)-fluorescence assay as described by Gillan *et al*.(1997) was used to assess the capacitation status of spermatozoa. The CTC staining solution was prepared prior to each experiment. It contained 750 µM CTC-HCl in stock

buffer (stored at 4 ^{o}C), 20 mM Tris, 130mM NaCl, and 5 mM L-cysteine (all reagents from Sigma, USA). A 50µl sample of spermatozoa suspension was placed in a light-protected eppendorf tube and an equal volume of CTC staining solution was added. After thorough mixing for 30 seconds, a 10µl sample of filtered glutaraldehyde (EM grade; 1% v/v in 1 M Tris, pH 7.8) was added to fix the spermatozoa. A 10µl sample of this uniformly mixed suspension was placed onto a clean microscope slide and 10 µl of 1.4-diazabicyclo 2.2.2] octane (DABCO, 0.22 M, Sigma, USA) dissolved in glycerol: PBS (9:1) was added to retard photobleaching. A coverslip was placed on the sample, and excess fluid was removed by compression and the edges of the coverslip were sealed with colourless nail varnish. The slides were examined at 40 x magnification with a fluorescence microscope (Leitz Wetzlar, Germany) and 100 spermatozoa evaluated, unless otherwise specified.

Three categories of capacitation status were identified:

- Uncapacitated spermatozoa (Figure 3.2). A bright band of yellow fluorescence present on the head and on the mid-piece of the spermatozoon.
- Capacitated acrosome-intact spermatozoa (Figure 3.3). A bright band of fluorescence was present on the anterior portion of the head and on the mid-piece whereas the post acrosomal region was non-fluorescent.
- Capacitated acrosome-reacted spermatozoa (Figure 3.4). A bright band of fluorescence was present only on the mid-piece and the head of the spermatozoon was non-fluorescent.

Figure 3.2 Chlortetracycline fluorescence staining patterns for uncapacitated spermatozoa. A bright band of yellow fluorescence was present on the head and on the mid-piece (arrows) of the spermatozoon.

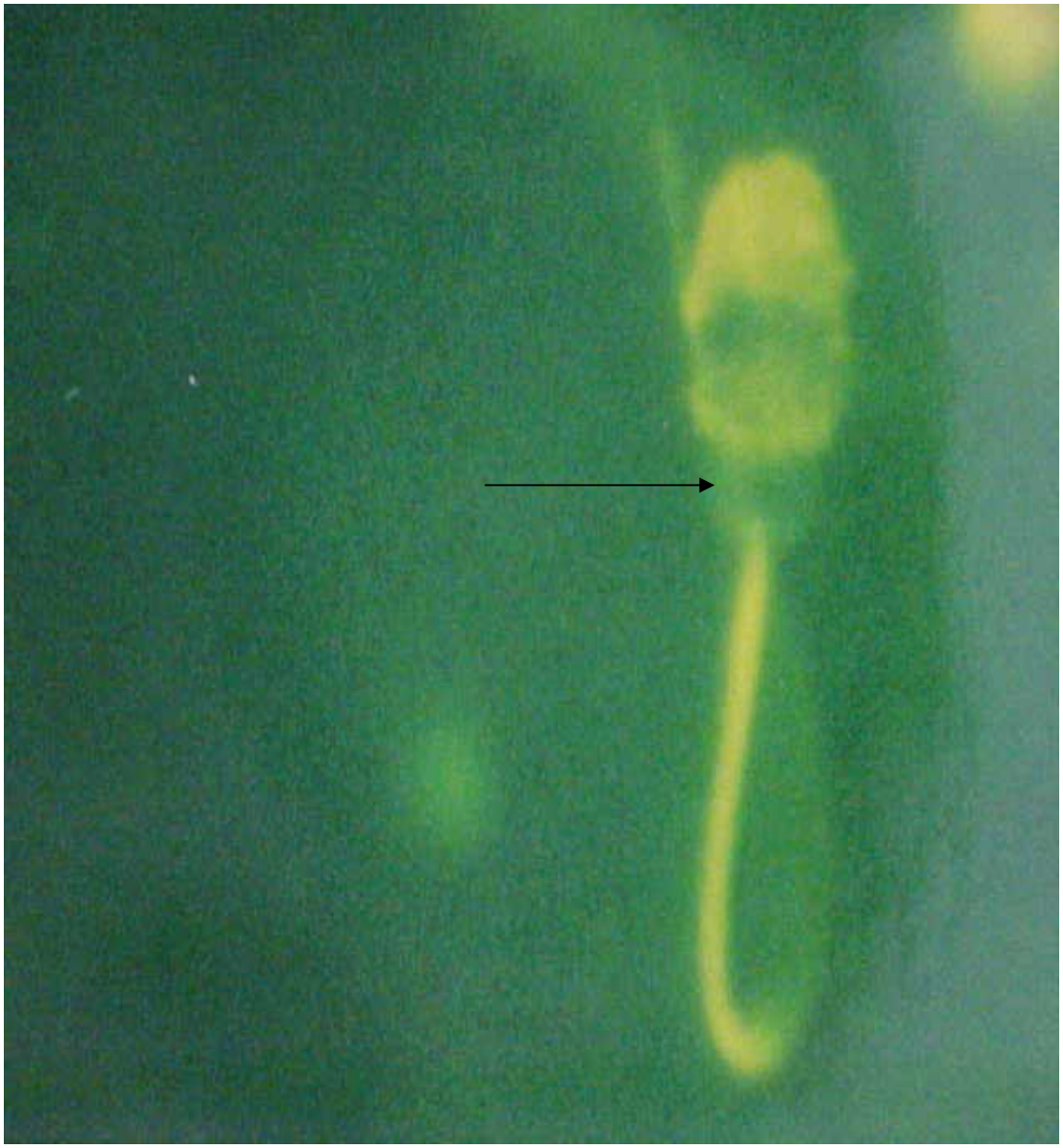


Figure 3.3 Chlortetracycline fluorescence staining patterns for capacitated acrosome-intact spermatozoa. The post acrosomal region was non-fluorescent (arrow).

Figure 3.4 Chlortetracycline fluorescence staining patterns for capacitated acrosome-reacted spermatozoa. A bright band of fluorescence was present only on the mid-piece (arrow) and the head of the spermatozoon was non-fluorescent.

3.10 Statistical Analyses

All data were analysed using SPSS software program (SPSS 11.0 Brief Guide, New Jersey, USA). The data were analyzed using analysis of variance (ANOVA) with one way classification, and the level of significance was considered to be $P \leq 0.05$ whereas the differences between means were tested by Least Significant Difference test. A more detailed description of statistical analyses used for different sets of data is included in the Materials and Methods sections of subsequent Chapters.

CHAPTER 4

IN VITRO MOTILITY, LONGEVITY AND CAPACITATION STATUS OF MERINO RAM SPERMATOZOA

4.1 Introduction

The motility and longevity of spermatozoa within the female reproductive tract are important factors if spermatozoa are to reach the oviducts and fertilise oocytes. Strong progressive motility is particularly important if spermatozoa are to pass through the mucosal folds and mucus of the cervix and the folds and mucus of the utero-tubal junction. The development of computer-aided semen analysis has taken away much of the subjective assessment of spermatozoa and enables the objective assessment of the detailed movement characteristics of spermatozoa under a variety of physiological and other experimental conditions. Examples of recent studies that have used computer-aided semen analysis to define the motility characteristics of spermatozoa include rams (Bag *et al*., 2002), boars (Vasquez *et al*., 2002), mice (Si and Olds-Clarke, 2000) and camels (Al-Qarawi *et al*., 2002).

Capacitation is a prerequisite that renders spermatozoa capable of achieving fertilization (Austin, 1951; Chang, 1951). Normally capacitation takes place in the female reproductive tract of animals and humans during the peri-ovulatory period but will also occur in a variety of artificial media without any contribution from the female (Yanagimachi, 1989).

Capacitation of spermatozoa *in vitro* has been widely investigated in a number of species under different circumstances including sheep (Gillan *et al*., 1997; Gomez *et al*., 1997; Gillan *et al*., 2000), rabbits (Hafez, 1980), goats (Kaul *et a*l., 1997), hamsters (Smith *et al*., 1998; Arnoult *et al*., 1999; Si and Olds-Clark, 2000; Kulanand and Shivaji, 2001), pigs (Wang *et al*., 1995; Mattioli *et al*., 1996; Green and Watson, 2001; Funahashi, 2002; Kaneto *et al*., 2002; Huo *et al*., 2002; Suzuki *et al.*, 2002), buffaloes (Kaul *et al.*, 2001; Kitiyanant *et al.*, 2002), horses (Rathi *et al*., 2001; Colenbrander *et al*., 2002; Pommer *et al*., 2002; Pommer and Meyers, 2002), and cattle (Byrd, 1981; Bracket *et al*., 1982; Parrish *et al*., 1988; Ijaz and Hunter, 1989; Parrish *et al*., 1989; McNutt and Killan, 1991; Fraser *et al*., 1995; Iqbal and

Hunter, 1995; Topper *et al.,* 1999; Coscioni *et al*., 2001; Kaneto *et al*., 2002; O'Flaherty *et al*., 2002).

Substances that have been used to facilitate the *in vitro* capacitation process of mammalian spermatozoa include Hepes-synthetic oviduct fluid (Gomez *et al*., 1997), Tyroide's albumin-lactate-pyruvate (TALP) medium (Parrish *et al*., 1988; Green and Watson, 2001), calcium ionophore A23187 (Byrd, 1981; Kitayanant *et al*., 2002), a high ionic strength medium (Brackett *et al*.,1982), serum albumin (Harrison *et al.,* 1982; Go and Wolf, 1985), heparin (Parrish *et al*., 1988; Kitayanant *et al*., 2002), caffeine (Niwa and Ohgoda, 1988), oviduct fluid (Parrish *et al*., 1989), oviductal epithelial cells (Ellington *et al*., 1991) and follicular fluid (McNutt and Killian, 1991).

Capaciated spermatozoa can be identified by several methods including the spermatozoa marker *Pisum sativum* agglutinin (Fabro *et al*., 2002) and chlortetracycline (Wang *et al*., 1995; Fraser *et al*., 1995; Gillan *et al*., 1997; Gomez *et al*., 1997; Gillan *et al*., 2000; Green and Watson, 2001; Kaul *et al*., 2001; Huo *et al*., 2002; O'Flaherty *et al*., 2002; Suzuki *et al*., 2002; Aires *et al.,* 2003).

The aim of the present study was to determine the effects of individual rams, incubation time and dilution rate in Hepes synthetic oviduct fluid (HSOF) medium on the motility, longevity and capacitation status of ram spermatozoa. In addition the influence of individual rams and incubation time of spermatozoa in TALP medium on the detailed motility, longevity and head of spermatozoa characteristics were analysed using a computer-aided semen analyser (CASA). The study also compared the effects of TALP medium and HSOF medium on the motility and velocity characteristics of spermatozoa.

4.2 Material and Methods

4.2.1 Animals

Eight rams (eartag numbers R1, R3, R5, R6, R9, R12, R13, R16) of proven fertility were used in this study. Semen was collected by electroejaculation (see Section 3.4). Rams were divided into two groups (Group I and Group II). In Group I (R1, R3, R5 R6), semen was collected three times at intervals of one to three weeks and spermatozoa analysed for motility, longevity (undiluted semen at 23^{o}C and diluted semen at 39^{o}C) and capacitation status. In Group II (R9, R12, R13, R16) semen was collected three times with an interval between each collection of one to two weeks, and analysed for motility, velocity and spermatozoa head morphology characteristics by using CASA. The interval between collection of semen and first analysis of spermatozoa in the CASA was about 5 minutes.

4.2.2 Sperm preparation and analysis

The fresh semen in Group I was diluted at four dilutions (1:25, 1:20, 1:15, 1:10) in Hepes buffered synthetic oviduct fluid (HSOF) (see Table 3.1) in microcentrifuge tubes (Eppendorf tubes). The diluted semen samples were held on a microscope warm stage set at 39 ^{o}C (LEC Instruments; Scoresby, Victoria). This temperature was selected in order to approximate the temperature within the reproductive tract of the ewe. A sample of semen was collected at 0, 4, 8 and 12 hours and the motility and capacitation status of spermatozoa determined. The capacitation status of spermatozoa was determined as described in Section 3.9.

In Group II, 50 µl of fresh semen from each semen sample was diluted in a microcentrifuge tube in 600 µl of TALP and HSOF medium respectively, incubated at 39 ^{o}C and the motility characteristics of spermatozoa determined with CASA as described in section 3.6.

4.2.3 Statistical analyses

All data were analysed using the SPSS software program (SPSS 11.0 Brief Guide, New Jersey, USA). Analysis of Variance (ANOVA) one way classification was used to determine the effect of rams, dilution rate and incubation time on the motility and longevity of spermatozoa. The effect of incubation time on motility, velocity and morphology characteristics of ram spermatozoa, and the differences in motility, velocity and morphology characteristics of rapid, medium and slow motile spermatozoa were analyzed using ANOVA one way classification. Differences between the effect of TALP and HSOF medium were tested by the Student t-test. Capacitation status was analysed by univariate, analysis of variance to determine the effects of rams, incubation time and dilution rate on the capacitation status. The level of significance was considered to be $P \leq 0.05$. The differences between means were tested by the Least Significant Difference test.

4.3 Results

4.3.1 Characteristics of Merino ram semen

The mean semen volume and motility of spermatozoa of Group I rams was 0.89 ml and 81.7% (Table 4.1), and the semen varied in color from milky to thick creamy. The mean longevity of undiluted semen at room temperature (23 ^{o}C) was 13.8 hours (range 2 to 30 hours) whereas the longevity of semen diluted in HSOF at 39 ^{o}C was 10.2 hours (range 6 to 12 hours).

The percentage of motile spermatozoa in incubated undiluted semen varied between rams (Figure 4.1 A). Two rams (R3 and R6) were similar with spermatozoa having a short period of motility whereas the other two rams (R1 and R5) had spermatozoa that were motile for a considerably longer period of time. However the motility of spermatozoa in semen diluted in HSOF and incubated at 39 ^{o}C was similar in all rams, and there was no significant difference between the rams (Figure 4.1-B). In addition there was no significant effect of the dilution rate on the motility and longevity of spermatozoa (Figure 4.2).

Table 4.1 The mean (± SEM) semen volume, motility of spermatozoa, semen colour and longevity of spermatozoa of the four rams in Group I.

	Semen volume (ml)	Sperm motility (%)	Semen colour	Sperm longevity (hours)	
				Undiluted semen	Diluted semen
Mean	0.89 ± 0.38	81.7 ± 11.9	3.5 ± 0.78	13.8 ± 12.8	10.2 ± 2.4
Range	(0.45 - 1.70)	(70 - 90)	(2 - 5)	(2 - 30)	(6 - 12)

4.3.2 Effect of incubation time on the motility and morphological characteristics of ram spermatozoa in TALP medium

Four rams (R9, R12, R13, R16) (Group II) were used in this section. The mean percentage of motile spermatozoa, and the percentage showing progressive and rapid motility at 0 time of incubation was 78.4%, 54.5% and 64.1% (Table 4.2), whereas at 120 minutes incubation the percentages had declined significantly to 29%, 12.5% and 15.4%, respectively. In contrast, the mean percentage of static spermatozoa significantly increased from 21.6% to 71.3% at 0 to 120 minutes incubation. The percentage of motile and rapidly motile spermatozoa at 0 and 30 minutes incubation was not significantly different, whereas the percentage of progressively motile spermatozoa at 0 and 30 minutes incubation was statistically different ($P \leq 0.05$).

The mean average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) at 0 time incubation were 91.2 μm/s, 72.2 μm/s and 134 μm/s and these values decreased significantly to 49 μm/s, 40.8 μm/s and 85.2 μm/s at 120 minutes incubation, respectively (Table 4.2). Average path velocity and VSL were similar at 0 and 30 minutes incubation but VCL had significantly declined by 30 minutes incubation. Amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and linearity (LIN), elongation and head area of spermatozoa at 0 time incubation were 5.5 μm, 19.5 Hz, 54.7%, 39.3% and 3.2 μm^2, respectively, but these parameters were not significantly influenced by incubation time.

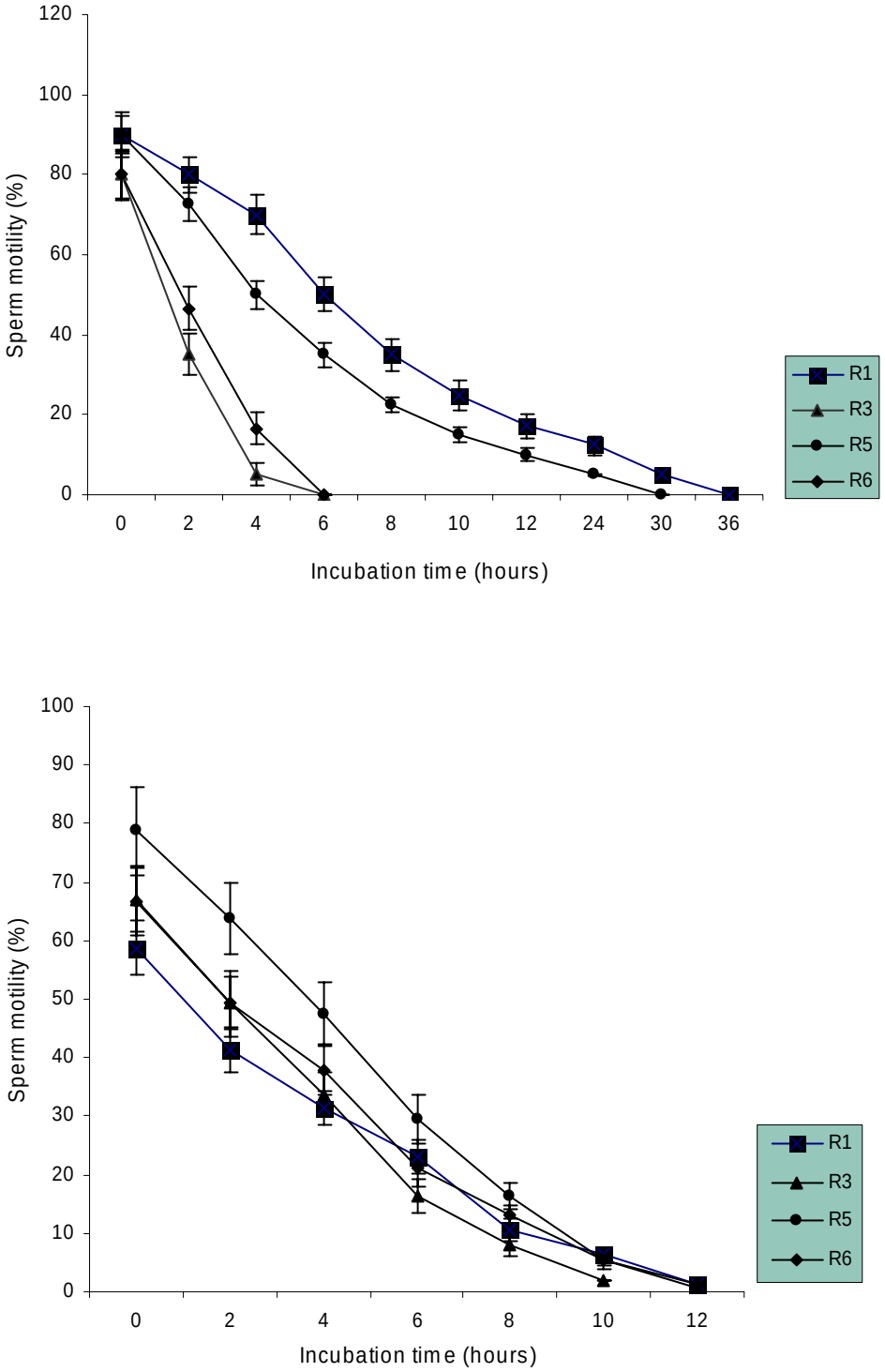


Figure 4.1 Effect of incubation time and rams (R1, R3, R5, R6) on the motility of spermatozoa in undiluted semen (Figure A) at room temperature (23 ºC) and semen diluted (Figure B) in HSOF medium at 39 ºC. The results are the mean (± SEM) of three replicates for each ram.

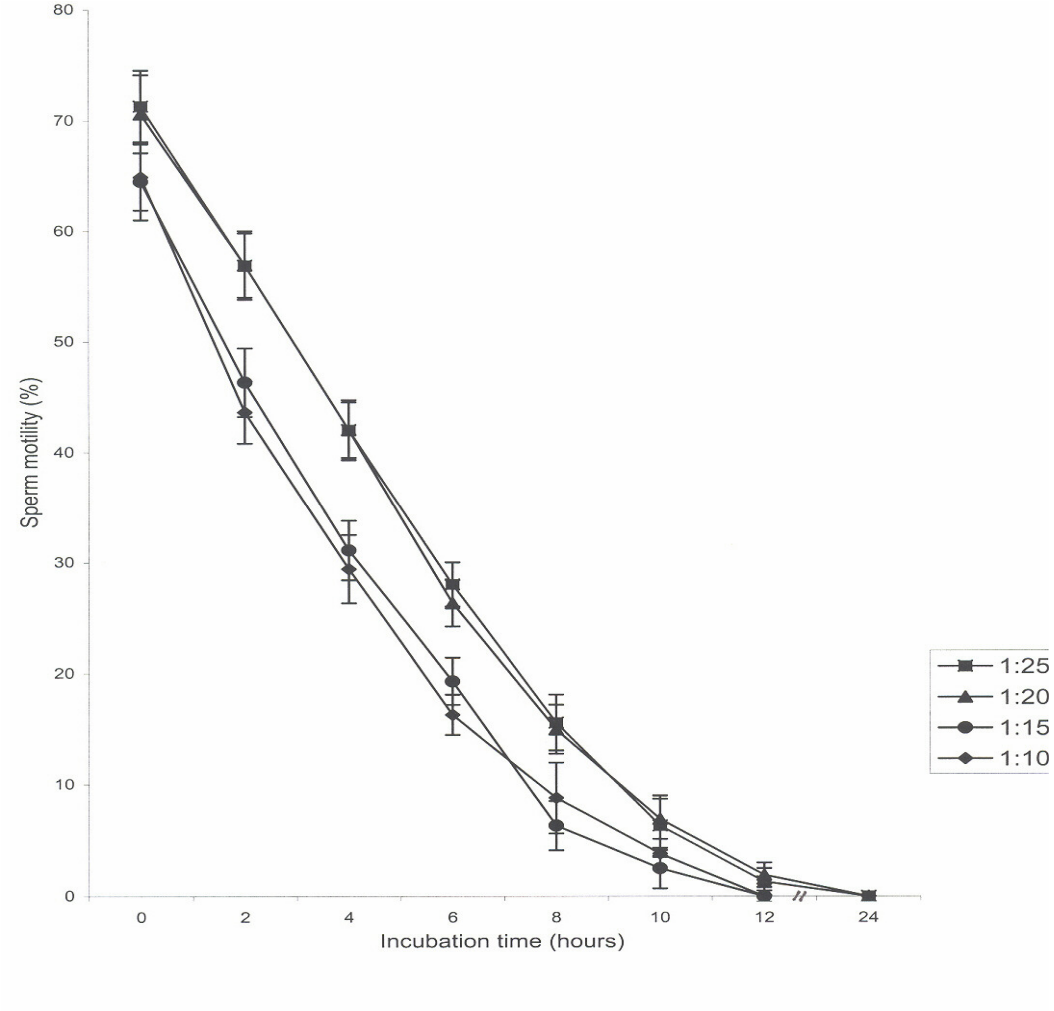


Figure 4.2 Relationship between incubation time and dilution rate of semen (1:25, 1:20, 1:15, 1:10) in HSOF medium on the motility of spermatozoa. The results are the mean (± SEM) of three replicates for each ram (R1, R3, R5, R6).

Table 4.2 Effect of incubation time in TALP medium at 39 °C on the motility and morphological characteristics of ram spermatozoa (mean ± SEM).

Parameters	Incubation time (minutes)				
	0	30	60	90	120
Number of spermatozoa analysed	274 ± 43	263 ± .35	259 ± 24	260 ± 27.8	236 ± 8.6
Motile (%)	78.4± 8.3[a]	70.8±3.9 [a]	51.9 ± 8.3 [b]	43.5 ± 7 [b]	29.0 ± 8.1[c]
Progressive (%)	54.5±11.4[a]	38.3 ± 3.9 [b]	31.8 ± 10.9[b]	22.2 ± 3.6[c]	12.5±2.5[d]
Velocity distribution:					
Rapid (%)	64.1±12.6[a]	48.3± 3.6[ab]	34.9± 12.1[bc]	28.3 ± 5.3[c]	15.4±4.3[d]
Medium (%)	7.2 ± 2.2	9.8 ± 1.4	9.4 ± 2.8	9.5 ± 1.5	6.8 ± 1.6
Slow (%)	6.8 ± 1.6	9.8 ± 1.4	7.3 ± 3.4	8.3 ± 4.9	6.9 ± 2.9
Static (%)	21.6±8.3 [a]	29.3 ± 3.9 [a]	48 ± 8.3[b]	54 ± 10.51[b]	71.3 ± 8.2[c]
Average path velocity (µm/s)	91.2± 8.4 [a]	71.2±13.4[ab]	63.3±17.3 [bc]	55.8±10.[cd]	49.0 ± 6.4[d]
Straight-line velocity (µm/s)	72.2±16.5[a]	61.1± 15.3 [a]	54 ± 18.5[ab]	49.9± 14.[ab]	40.8 ± 6.2 [b]
Curvilinear velocity (µm/s)	134± 16.8[a]	100 ± 23.9[b]	93.3 ±16.5 [b]	85.2±11.9[b]	85.2± 11.9[b]
Amplitude of lateral head displacement (µm)	5.5 ± 0.58	4.9 ± 0.38	4.5 ± 0.9	4.2 ± 0.48	4.3 ± 0.58
Beat cross frequency (Hz)	19.5 ± 1.3	20.5 ± 1.9	21.4 ± 3.3	20.3 ± 3.2	19.6 ± 3.2
Straightness (%)	70.3 ± 10.9	77.8 ± 5.8	76.6 ± 7.4	75.8 ± 4.4	74.7 ± 3.2
Linearity (%)	54.7 ± 8.8	53.1 ± 9.1	50.5 ± 10.7	49.6 ± 6	47.6 ± 3.8
Elongation (%)	39.3 ± 0.9	39.8 ± 2.9	39.6 ± 2.4	40 ± 1.6	37.3 ± 3.5
Area (μm^2)	3.2 ± 0.48	3.4 ± 0.28	3.4 ± 0.58	3.3 ± 0.86	3.1 ± 0.77

Each time value represents the mean of 12 replicates (from 4 rams with 3 semen collections).
Data with different superscripts within a row were significantly different ($P \leq 0.05$).
Data in a row with no superscripts were not significantly different.

While there was no significant difference between rams in the percentage of motile spermatozoa at 0 incubation time, R9 had a significantly higher percentage of progressively motile and rapidly motile spermatozoa (Figure 4.3). However this apparent superiority of R9 was not maintained through the 120 minutes of incubation and there was no consistent significance difference between the rams.

Average path velocity, VSL and VCL values decreased during incubation of spermatozoa from all rams but there was no consistent pattern of differences between rams except that R9 had consistently significantly lower values at 30, 60 and 90 minutes of incubation (Figure 4.4). Incubation time and rams did not significantly influence the values for ALH, BCF and STR (Figure 4.5) or linearity and elongation of spermatozoa (Figure 4.6) although spermatozoa from R12 had a significantly larger head area at 0, 60, 90 and 120 minutes of incubation.

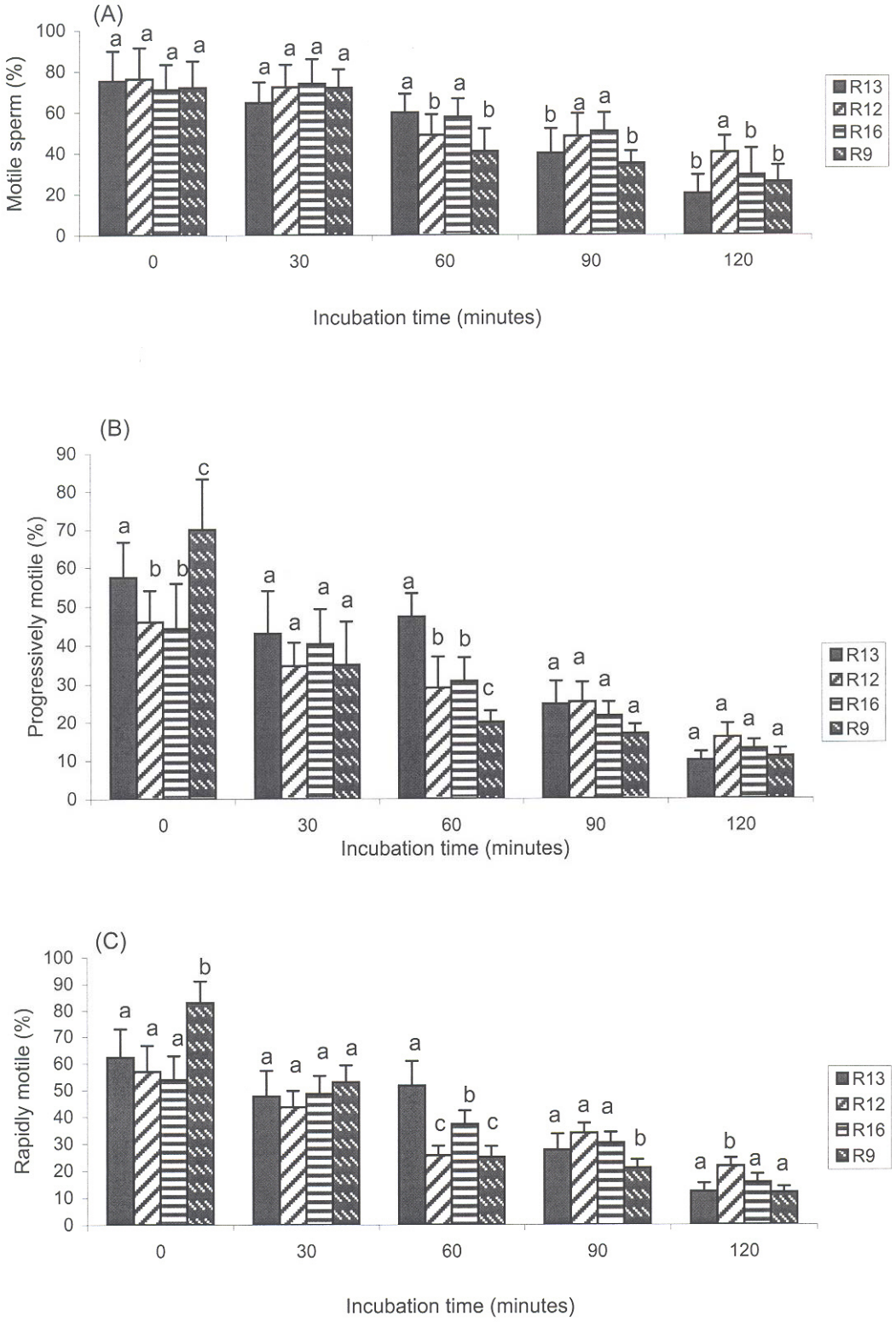


Figure 4.3 Effect of incubation in TALP medium at 39 ºC on the percentage of motile (Figure A), progressively motile (Figure B) and rapidly motile (Figure C) spermatozoa from rams (R13, R12, R16, R9) (mean ± SEM). Different letters above bars indicate significant differences ($P \leq 0.05$) within each incubation time.

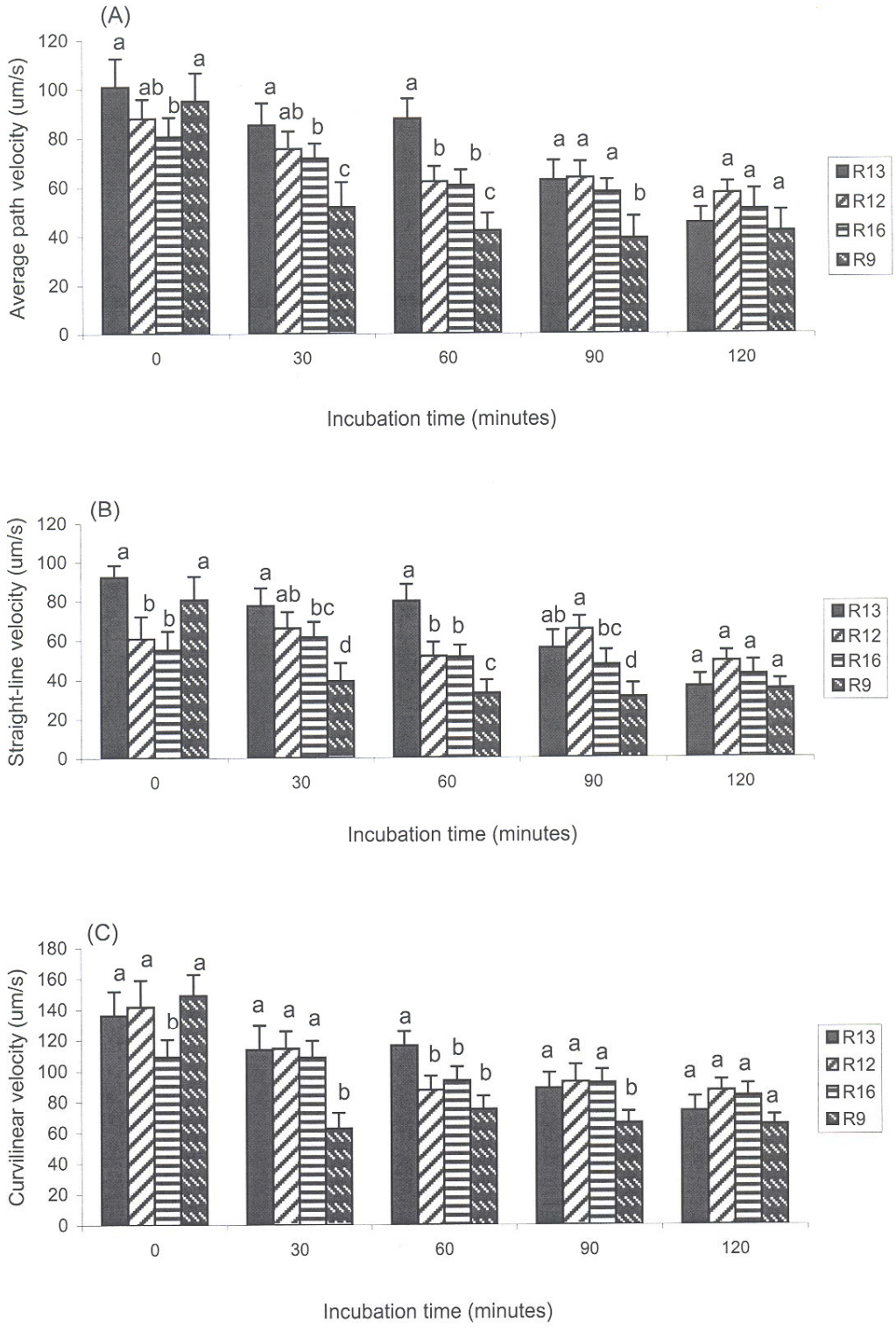


Figure 4.4 Effect of incubation in TALP medium at 39 °C on the average path velocity (Figure A), straight-line velocity (Figure B) and curvilinear velocity (Figure C) of spermatozoa from rams (R13, R12, R16, R9) (mean ± SEM). Different letters above bars indicate significant differences ($P \leq 0.05$) within each incubation time.

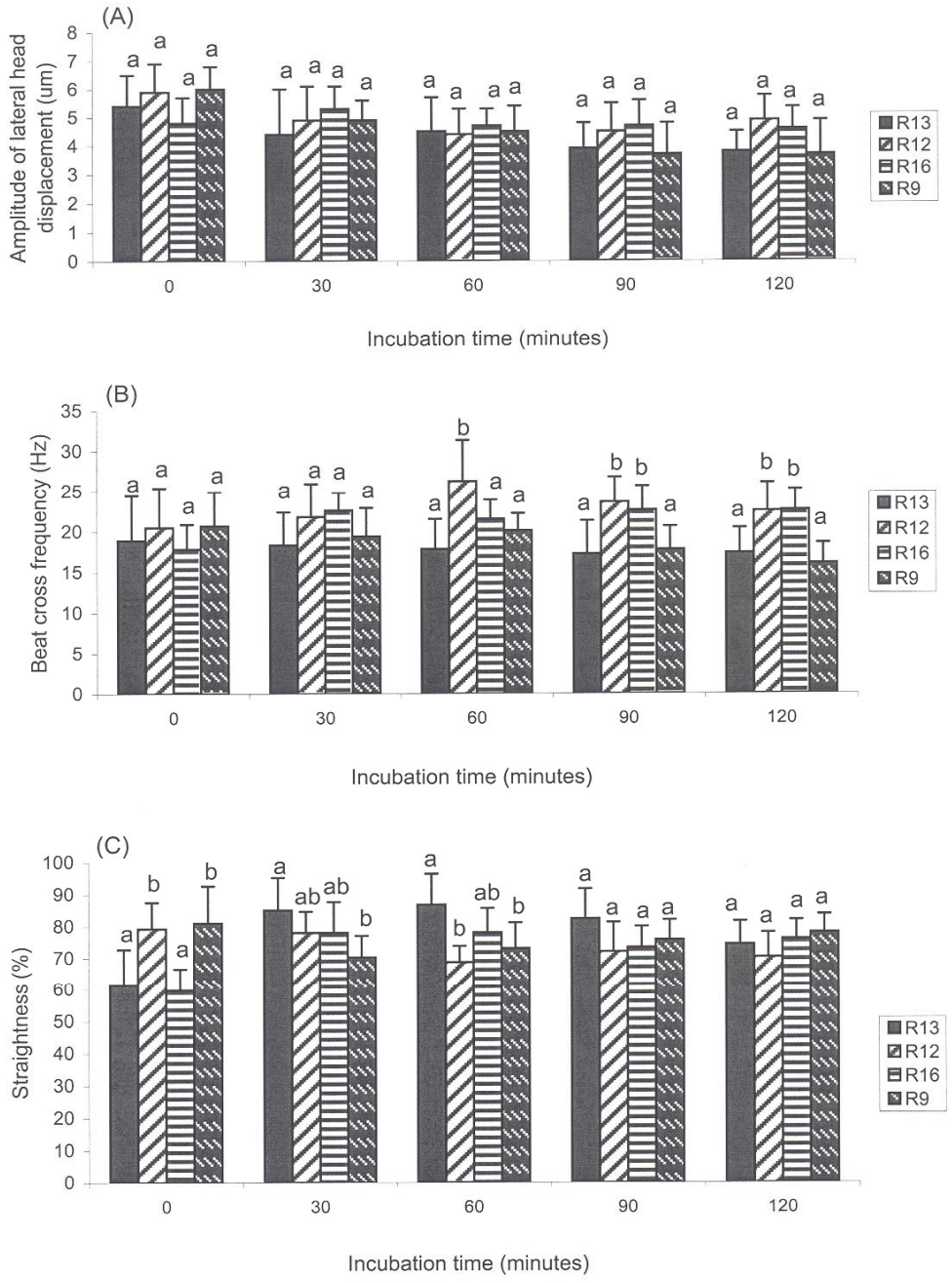


Figure 4.5 Effect of incubation in TALP medium at 39 ºC on the amplitude of lateral head displacement (Figure A), beat cross frequency (Figure B) and straightness (Figure C) of spermatozoa from rams (R13, R12, R16, R9) (mean ± SEM). Different letters above bars indicate significant differences ($P \leq 0.05$) within each incubation time.

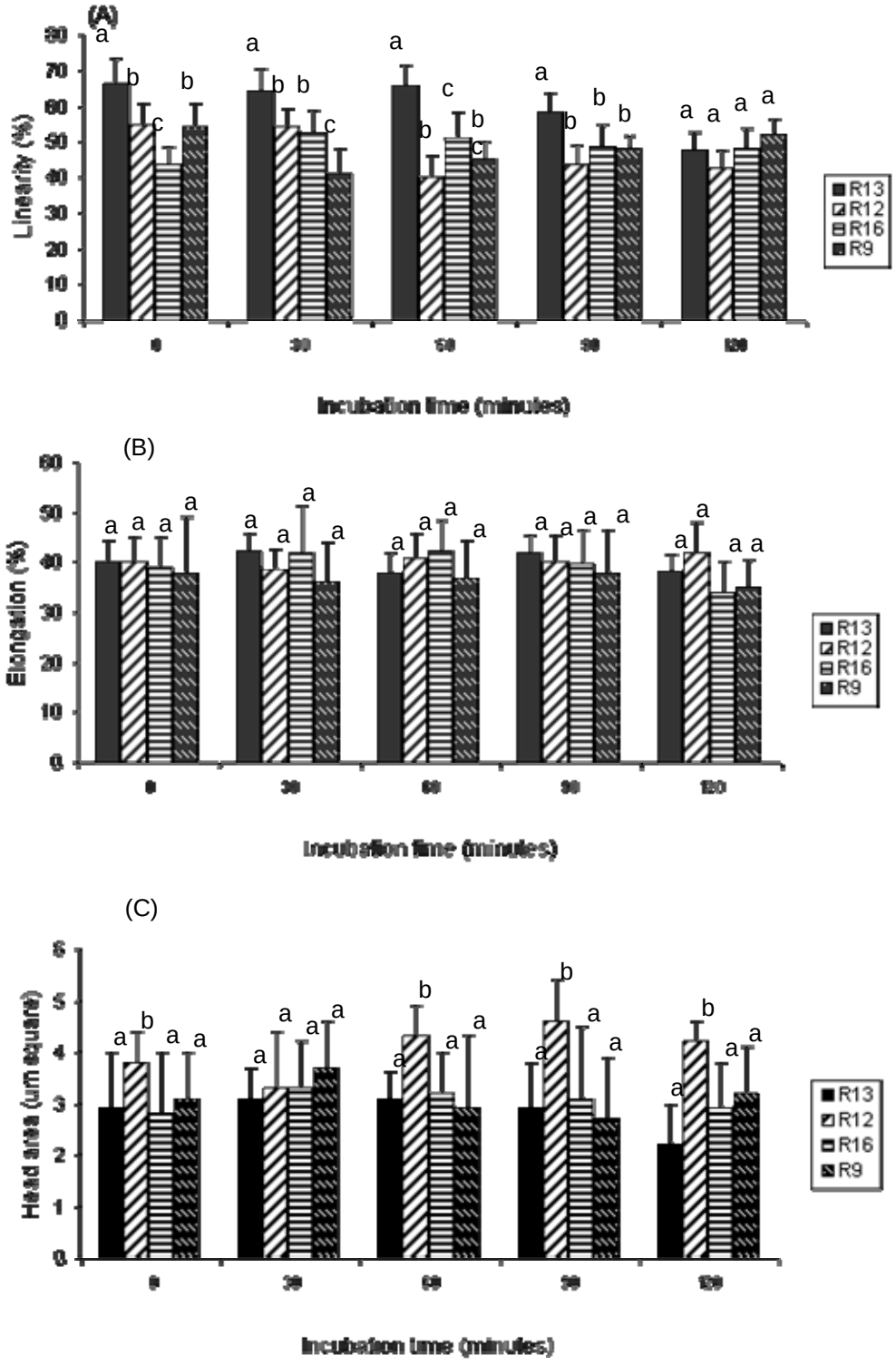


Figure 4.6 Effect of incubation in TALP medium at 39 ºC on the linearity (Figure A), elongation (Figure B) and head area (Figure C) of spermatozoa from rams (R13, R12, R16, R9) (mean ± SEM).Different letters above bars indicate significant differences ($P \leq 0.05$) within each incubation time.

4.3.3 Distribution of ram spermatozoa into velocity and movement groups after incubation in TALP medium

The data on the velocity measurements of ram spermatozoa after incubation in TALP medium for 0, 30, 60, 90 and 120 minutes (Figure 4.3 – 4.6) were examined in greater detail. The results from the four rams (R9, R12, R13 and R16) were pooled and the data analysed based on the distribution of spermatozoa into velocity and movement groups from very slow to very fast (Figure 4.7 – 4.9).

Broadly speaking, the longer the spermatozoa were incubated, the slower the velocity path as determined by the average path velocity, straight-line velocity, curvilinear velocity and amplitude of lateral head displacement. For example, at 0 time incubation, 6.8 ± 2.3% of spermatozoa were in the slowest average path velocity group of 0-20 μm/s and by 120 minutes incubation, the percentage had increased to 27.4 ± 9.1% (Figure 4.7 A). Similarly for straight-line velocity, 14.9 ± 4.3% spermatozoa were in 0-20 μm/s group at the beginning of incubation and the percentage had increased to 47.5 ± 8.2% at 120 minutes incubation (Figure 4.7 B).

The amplitude of lateral head displacement of spermatozoa decreased during incubation. For example, at the commencement of incubation 20.5 ± 4.5% of spermatozoa were in the 2-4 μm group and after 120 minutes incubation 49.3 ±11.4% spermatozoa were in this group (Figure 4.9 A). However, the distribution of the beat cross frequency of spermatozoa was not significantly influenced by the incubation time (Figure 4.9 B).

4.3.4 Distribution of spermatozoa into velocity and movement groups

For ease of presentation, the data of the velocity and movement of spermatozoa from the four rams (R9, R12, R13, R16) determined immediately after dilution of semen in TALP medium was pooled. The percentage of spermatozoa that were distributed into various velocity and movement groups is shown in Figure 4.10 and Figure 4.11. With respect to curvilinear velocity, 20 ± 4.1% were in the rapid group of 180 + μm/s and the remainder of the spermatozoa were distributed across all the velocity groups with the lowest percentage (1.8 ± 0.4 %) being in the slowest group of 0-20

μm/s (Figure 4.10 A). A substantial percentage of spermatozoa (17.4 ± 1.8%) had the lowest straight-line velocity whereas about 45% had a straight-line velocity of between 80 and 140 μm/s. About 50% of spermatozoa had an average path velocity of between 80 and 140 μm/s (Figure 4.10 A). Also, about 50% of spermatozoa have a straightness of 90-100%, with about 45% distribution of elongation between 20-40%. There was a wide distribution of linearity with no more than 14% of spermatozoa within a group (Figure 4.10 B).

About 80% of spermatozoa have an amplitude of lateral head displacement of between 2 and 8 μm (Figure 4.11 A) and the majority of spermatozoa had a beat cross frequency of 0-6 Hz (Figure 4.11 B). However, the remainder of spermatozoa had beat cross frequencies that were distributed over a wide range.

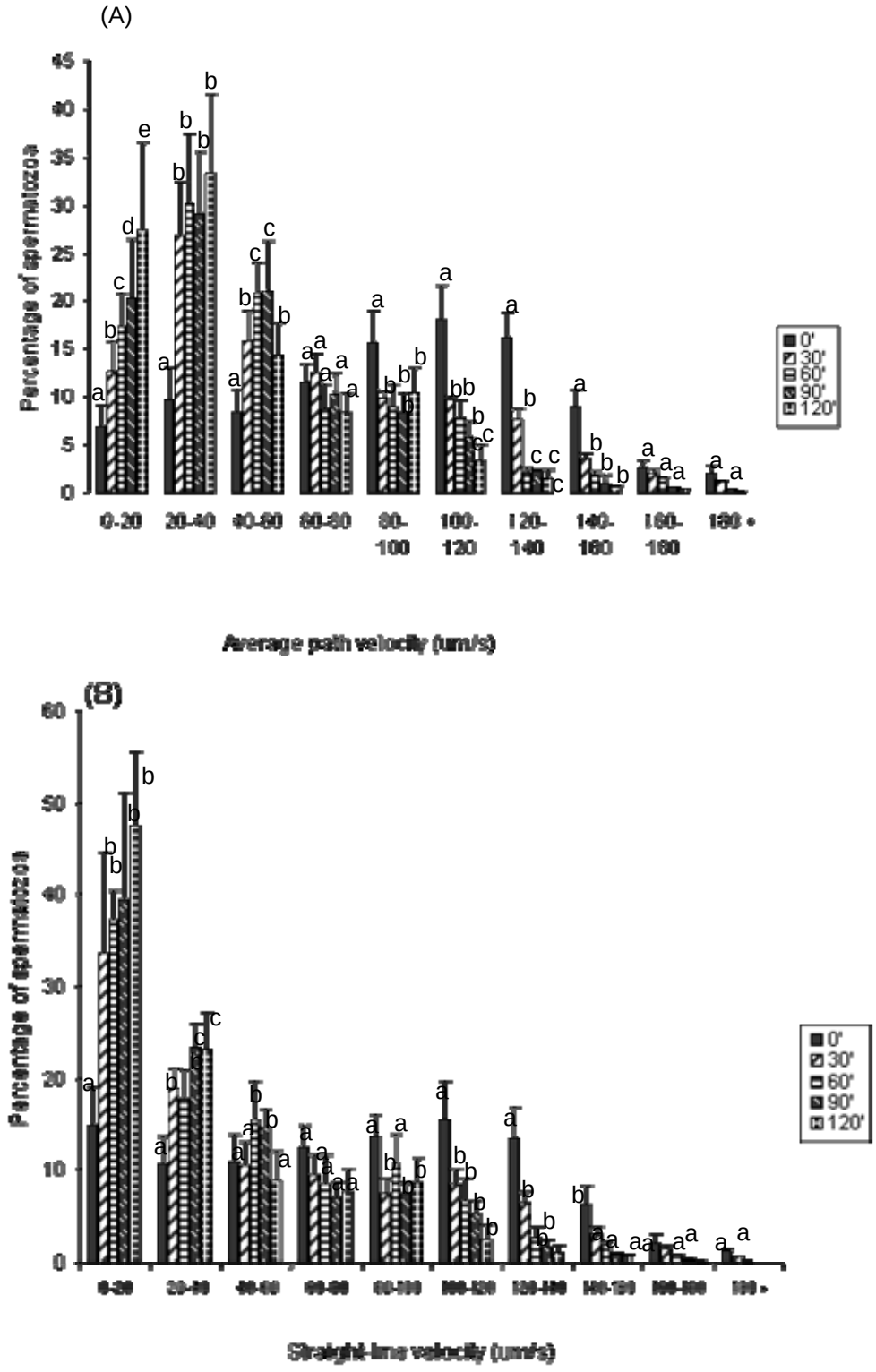
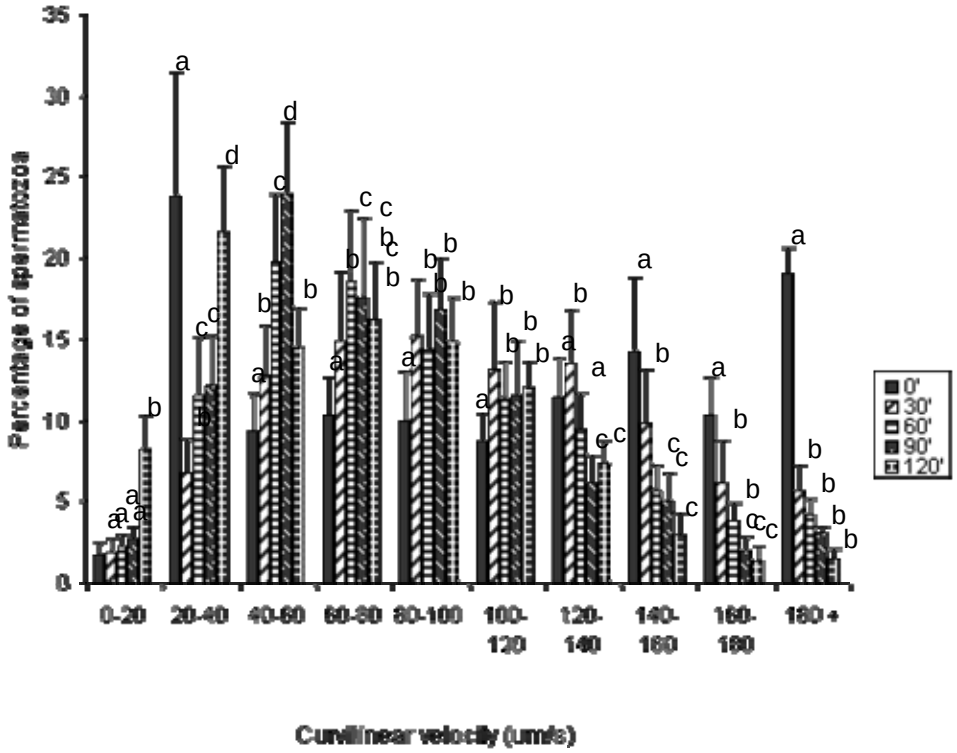


Figure 4.7 Distribution of spermatozoa (mean ± SEM) into velocity groups for average path velocity (Figure A) and staight-line velocity (Figure B) after incubation in TALP medium for 0, 30, 60, 90 and 120 minutes. Different letters above bars indicate significant differences ($P \leq 0.05$) within each velocity group.

(A)



(B)

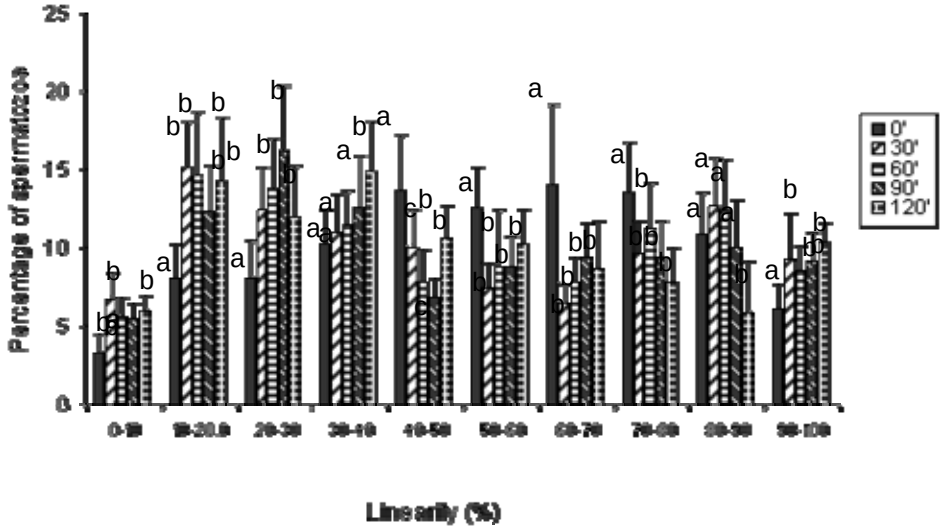


Figure 4.8 Distribution of spermatozoa (mean ± SEM) into velocity groups for curvilinear velocity (Figure A) and linearity (Figure B) after incubation in TALP medium for 0, 30, 60, 90 and 120 minutes. Different letters above bars indicate significant differences ($P \leq 0.05$) within each group.

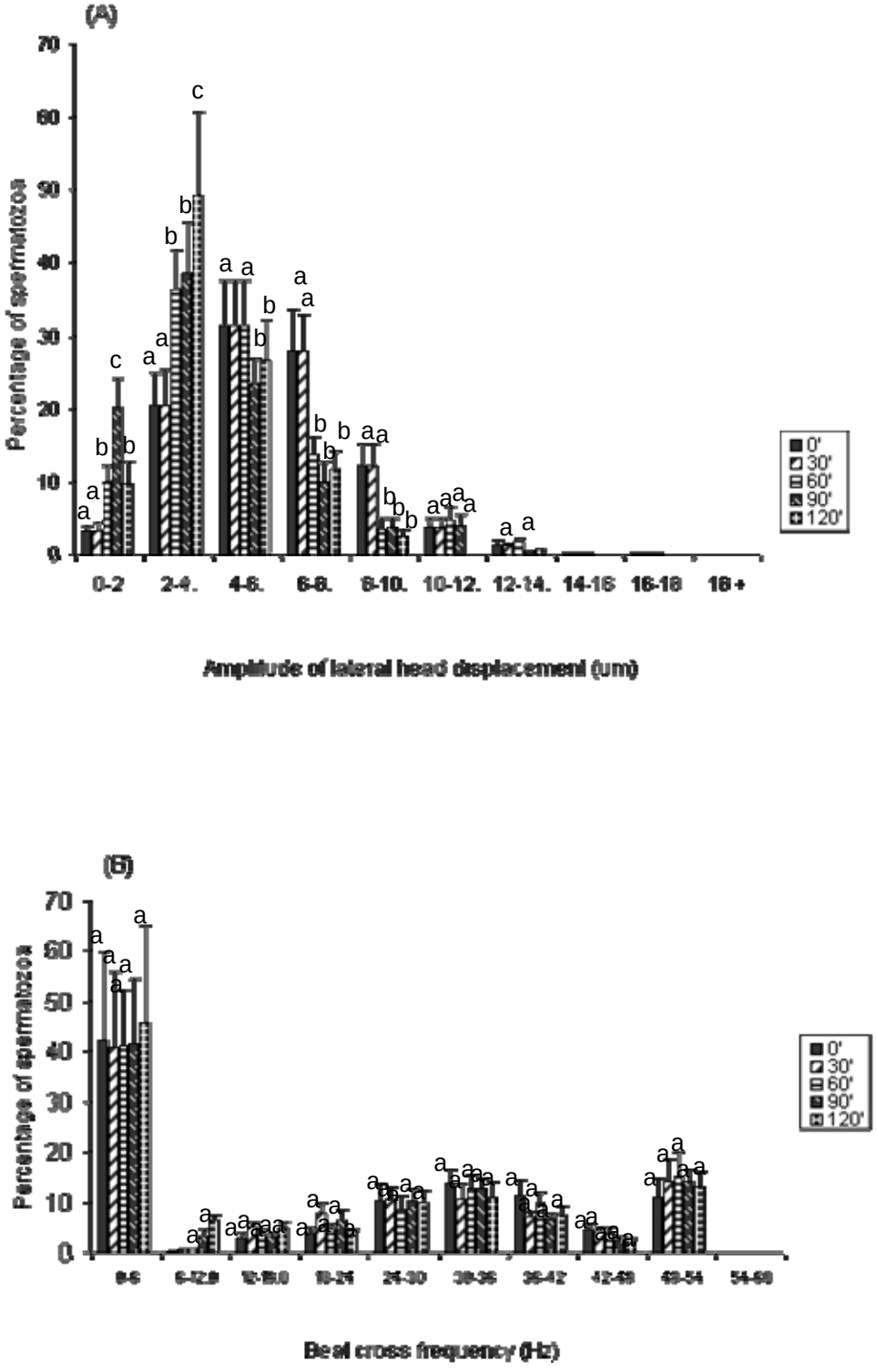


Figure 4.9 Distribution of spermatozoa (mean ± SEM) into groups for amplitude of lateral head displacement (Figure A) and beat cross frequency (Figure B) after incubation in TALP medium for 0, 30, 60, 90 and 120 minutes. Different letters above bars indicate significant differences ($P \leq 0.05$) within each group.

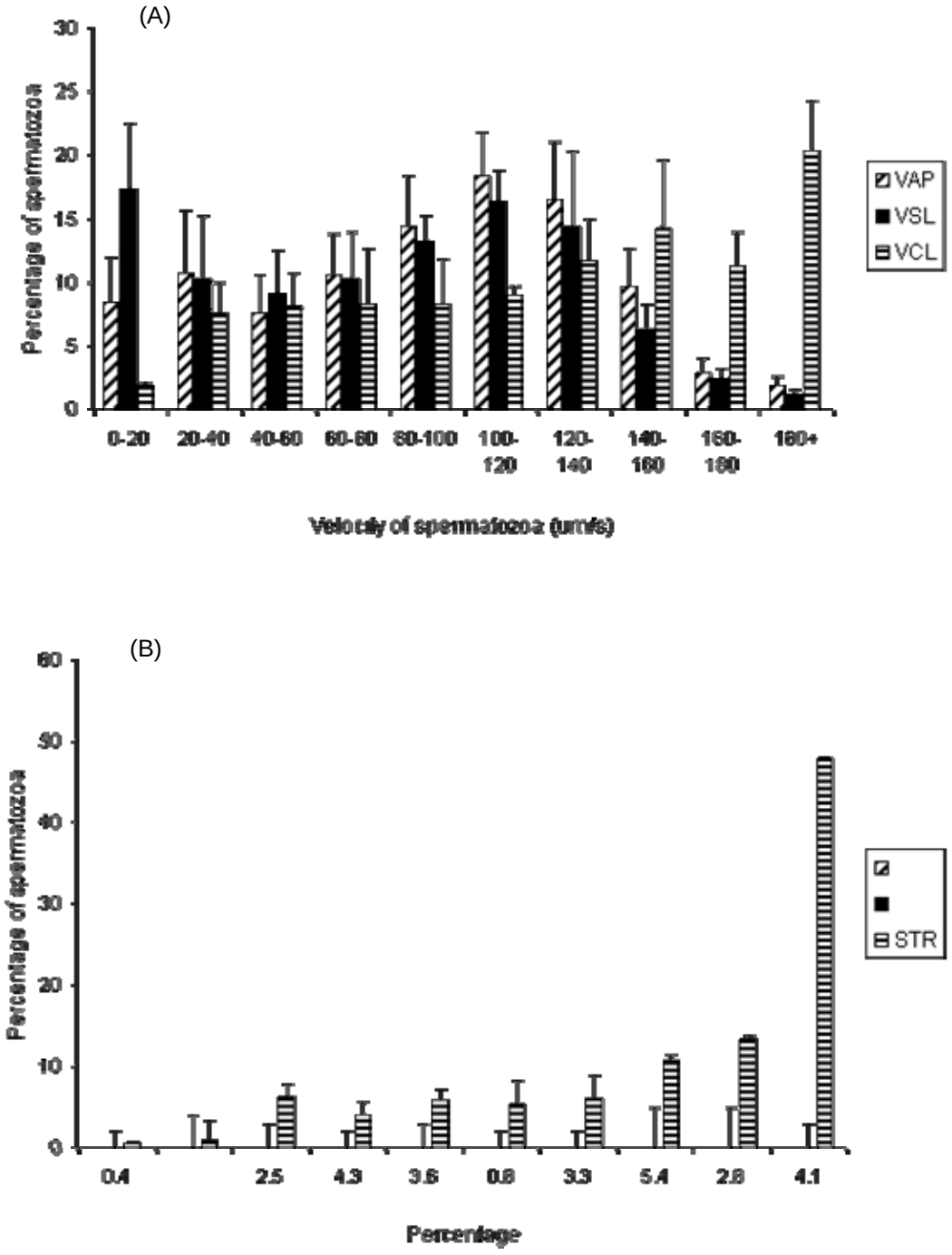


Figure 4.10 Distribution of spermatozoa (mean ± SEM) into velocity groups for the average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) (Figure A) and straightness (STR), linearity(LIN) and elongation (ELO) (Figure B) for rams (R9, R12, R13, R16). These were three semen collections for each ram and The semen was diluted in TALP medium at 39 ºC.

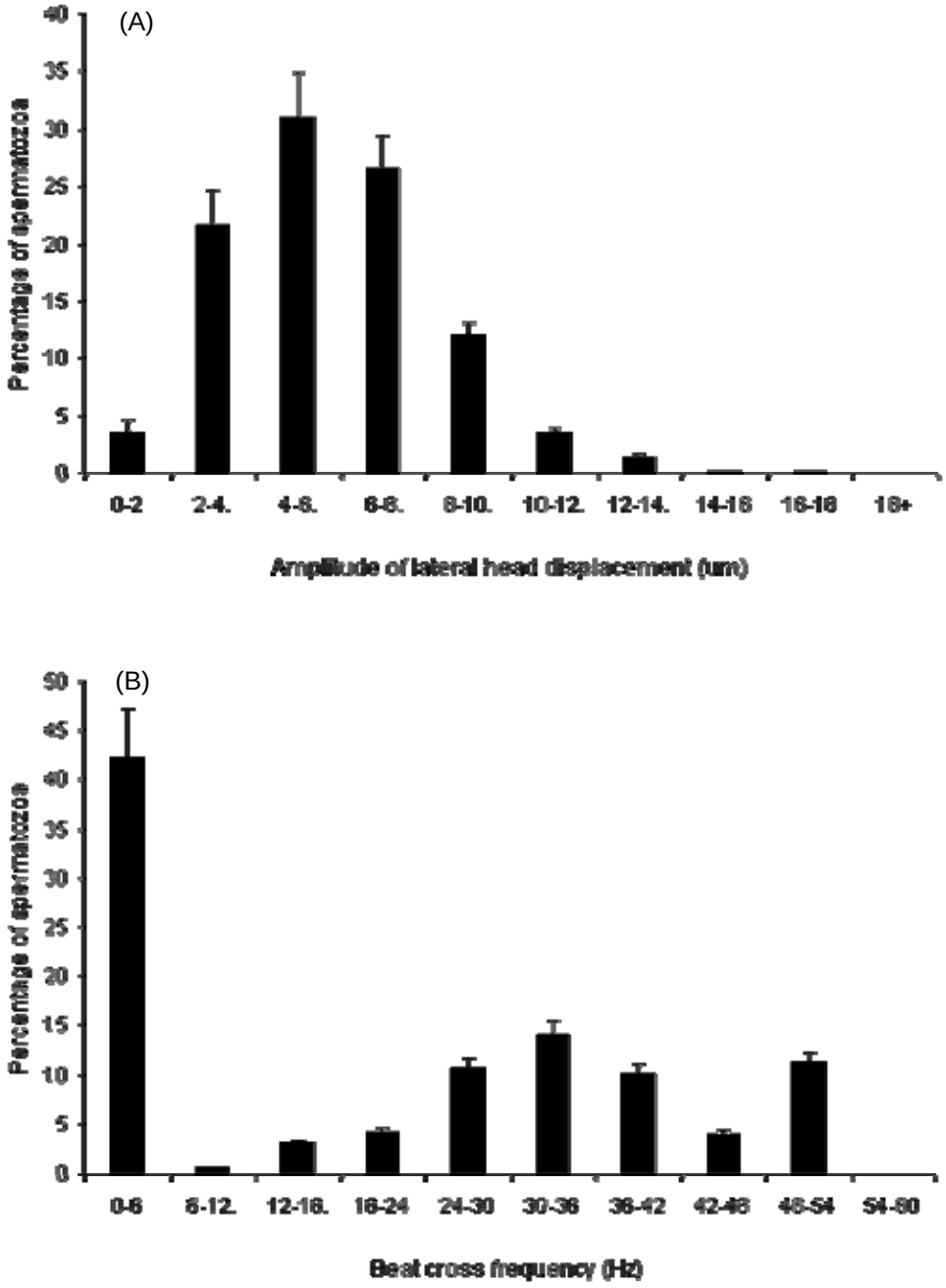


Figure 4.11 Distribution of spermatozoa (mean ± SEM) into groups for amplitude of lateral head displacement (ALH) (Figure A) and beat cross frequency(BCF) (Figure B) for rams (R9, R12, R13, R16). There were three semen collections for each ram and the semen was diluted in TALP medium at 39 ºC.

4.3.5 Comparison between TALP and HSOF medium on motility and velocity characteristics of ram spermatozoa *in vitro*

A comparison of the effect of dilution of spermatozoa in TALP and HSOF medium on the motility and velocity characteristics of ram spermatozoa is presented in Table 4.3. The percentage of progressively motile and rapidly motile spermatozoa as well as average path velocity and straight-line velocity in HSOF medium was greater ($P \leq 0.05$) than in TALP medium, but there was no significant difference in the other parameters.

Table 4.3 Effect of dilution in either TALP or HSOF medium on the motility and velocity characteristics of ram spermatozoa *in vitro* (mean ± SEM).

Parameters	TALP	HSOF
Motile (%)	91.9 ± 9.1	93.4 ± 5.4
Progressive (%)	48.9 ± 14.5[a]	54.6 ± 17.5[b]
Rapid (%)	65.4 ± 15.6[a]	72.8 ± 18.6[b]
Average path velocity (μm/s)	67.1 ± 16.3[a]	76.3 ± 18.4[b]
Straight-line velocity (μm/s)	52.8 ± 14.7[a]	60.9 ± 19.1[b]
Curvilinear velocity (μm/s)	112.4 ± 22.8	115.4 ± 28.7
Amplitude of lateral head displacement (μm)	5.7 ± 0.6	5.8 ± 0.6
Beat cross frequency (Hz)	19.7 ± 4.3	19.3 ± 3.9
Straightness (%)	73.6 ± 6.4	73.9 ± 7.3
Linearity (%)	46.1 ± 7.4	47.3 ± 9.5

Each time value represents the mean of five rams (R5, R9, R12, R13, R16) with five replicates for each ram. Data with different superscripts within each row were significantly different ($P \leq 0.05$).

4.3.6 Capacitation of spermatozoa after *in vitro* culture in HSOF medium

Immediately after dilution of semen in HSOF medium, most spermatozoa (93.5 ± 2.2 %) were not capacitated with a small percentage (6.4 ± 3.1%) being capacitated and acrosome-intact. During the 12 hours of incubation, progressively more spermatozoa became capacitated such that at the end of the incubation, 30.8 ± 2.5% were uncapacitated, 40.9 ± 0.9% were capacitated acrosome-intact and 29 ± 2.5% were capacitated acrosome-reacted (Figure 4.12). There was no significant effect of dilution on the capacitation rate (Figure 4.13).

There were differences between rams in the capacitation profile. This difference was present between four and six hours of incubation where R5 had significantly more capacitated acrosome-intact (Figure 4.14 A) and between four and 10 hours of incubation where R5 had significantly more capacitated acrosome-reacted spermatozoa than the other rams (Figure 4.14 B).

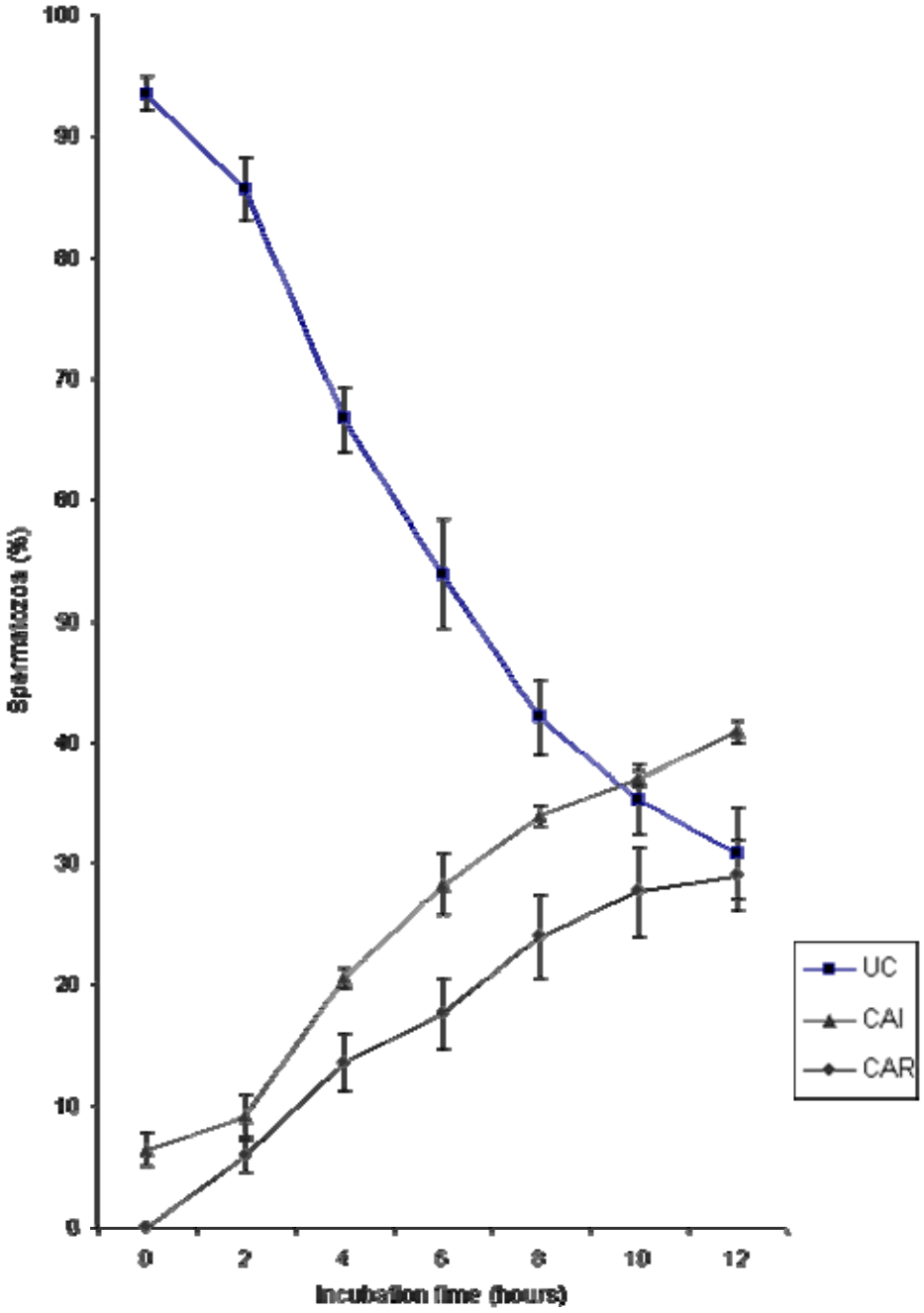


Figure 4.12 Relationship between uncapacitated (UC), capacitated acrosome-intact (CAI) and capacitated acrosome-reacted (CAR) ram spermatozoa during *in vitro* culture in HSOF medium. The results are the mean (± SEM) for four rams (R1, R3, R5, R6) with three replicates for each ram.

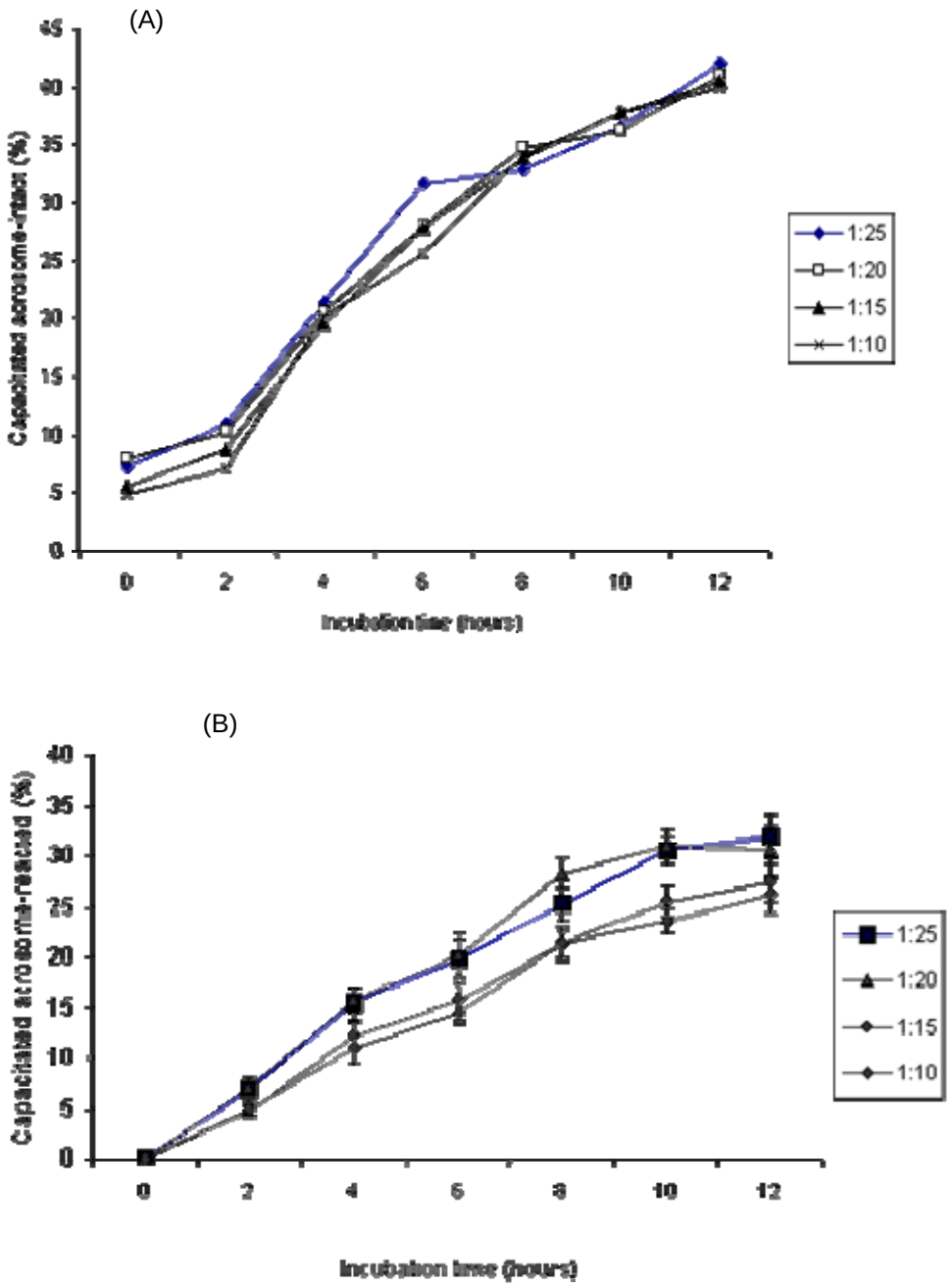


Figure 4.13 Influence of *in vitro* incubation time and dilution rate (1:25, 1:20, 1:15, 1:10)of semen on the percentage of spermatozoa that had undergone capacitation but were acrosome-intact (Figure A) and that had undergone the acrosome reaction (Figure B). The results are the mean (± SEM) of three replicates for each ram (R1, R3, R5, R6).

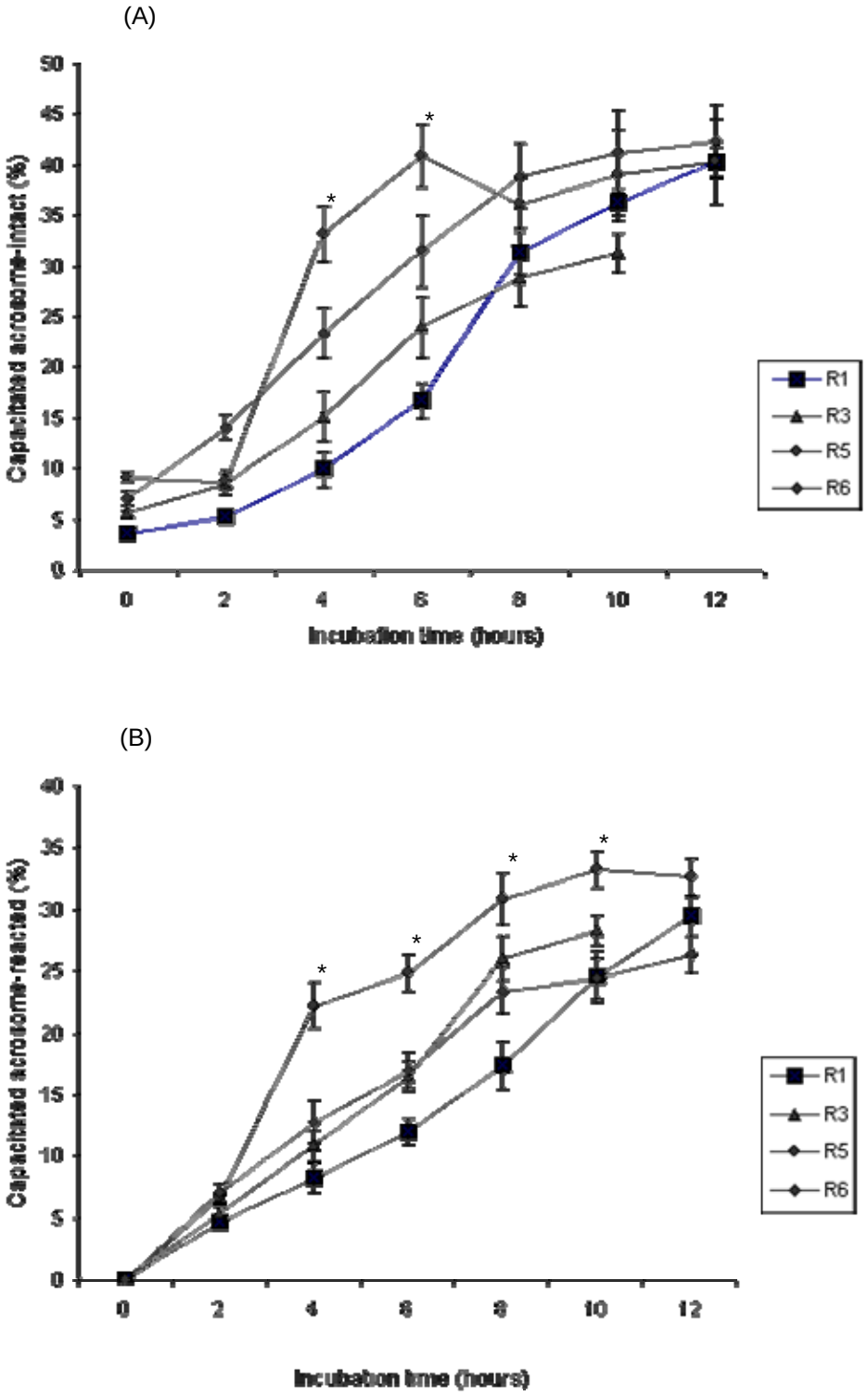


Figure 4.14 The mean (± SEM) percentage of capacitated acrosome-intact (Figure A) and capacitated acrosome-reacted (Figure B) spermatozoa from four rams (R1, R3, R5, R6) during *in vitro* culture in HSOF medium. There were three replicates for each ram. * Indicates a significant difference ($P \leq 0.05$) between R5 and the other rams.

4.4 Discussion

The studies reported in this Chapter were primarily designed to establish the techniques for analysis of ram spermatozoa and to provide baseline data on a range of motility parameters using the computer-aided semen analyser.

It has been recognised for sometime that mammalian spermatozoa have limited ability to survive in undiluted seminal plasma (Hamner, 1970; Ritar and Salamon, 1982; Ashworth *et al*., 1994; Paulenz *et al*., 2002). While there are factors in seminal plasma that have a detrimental effect on the viability of spermatozoa (Dott *et al*., 1979), exposure of semen to air increases the metabolic activity of spermatozoa in turn reducing the viability of spermatozoa because of lactic acid production and reduction of pH (Evans and Maxwell, 1987). The reduction of viability of ram spermatozoa in undiluted semen as measured by the motility of spermatozoa was confirmed in the studies reported in this Chapter. An interesting observation was that there were marked differences between rams, suggesting that some rams have specific substances in seminal plasma that could be detrimental to the survival of spermatozoa incubated at 23 ^{o}C. This detrimental effect in some rams was apparently lost when the semen was diluted and incubated in HSOF medium, although spermatozoa in HSOF medium were not able to survive as long as spermatozoa in undiluted semen for two of the four rams that were studied.

While a number of research workers have used CASA to examine motility characteristics of frozen-thawed ram spermatozoa (Bag *et al*., 2002; Gil *et al.,* 2003) there is limited information in the literature of detailed motility characteristics of spermatozoa from fresh semen except for the definition of hyperactivity of ram spermatozoa (Mortimer and Maxwell, 1999). Nevertheless, the results for VCL, VSL, VAP, linearity and beat cross frequency of spermatozoa diluted in TALP medium and immediately examined are similar to the result reported for Merino rams by Lambrechts *et al.* (2000).

The data on the distribution of velocity and movement of ram spermatozoa indicates the semen consists of a heterogenous population of spermatozoa. Other workers have used CASA to define sub-populations of spermatozoa in the boar, stallion and Gazelle (Abaigar *et al*., 1999; Thurston *et al*., 1999; Quintero-Moreno *et al*., 2003).

It is not clear how and why sub-populations are formed and which of the sub-populations contribute spermatozoa that fertilise oocytes *in vivo.*

The other piece of information that the CASA analysis revealed was that there were differences between rams in the head area of spermatozoa. One ram (R12) had a significantly larger head area than the other rams but this difference did not consistently reflect any differences in the velocity parameters. However, the calculation by CASA of head area may not be entirely accurate as the values are calculated simultaneously with the assessment of molitity. Nevertheless, manual calculations of the width and length of spermatozoa reported later in the thesis (page 107) did correlate with the results derived by CASA. The results on head area are interesting because other workers have identified small sperm heads with better fertility in boars (Hirai *et al.,* 2001). In cattle, Ostermeier *et al.* (2001) found that head shape of spermatozoa of highly fertile bulls was more elongated and tapered than those of lower fertility.

As expected, the majority of spermatozoa in a fresh semen sample were uncapacitated and following incubation in a physiological medium, the percentage of capacitated spermatozoa increased with incubation time. What determines why some spermatozoa are capacitated at say four hours after insemination and others are uncapacitated after 12 hours of incubation is not known but presumably relates to the fact that there is a heterogenous population of spermatozoa in a semen sample. In addition, there were differences between rams in the capacitation rate particularly between four to 10 hours of incubation.

In conclusion, these studies have established baseline information on the velocity and movement characteristics of ram spermatozoa as well as the establishment of procedures for the detection of the capacitated spermatozoa using the chlortetracycline assay.

CHAPTER 5

THE INFLUENCE OF CLIMATIC FACTORS AND THE VARIATION IN SEMEN PARAMETERS BETWEEN AND WITHIN MERINO RAMS AS DETERMINED BY COMPUTER-AIDED SEMEN ANALYSIS

5.1 Introduction

Reproduction in sheep can be influenced by photoperiod. This effect is most pronounced in the temperate areas of the world and is less pronounced or not evident in tropical environments. The sheep is classified as a short-day breeder. Increasing amounts of melatonin secreted by the pineal gland because of the lengthening periods of darkness and shortening day-light hours drive the increased synthesis and secretion of gonadotrophin releasing hormone from the hypothalamus. As a consequence, there is increased gonadal activity in the ram and ewe. Many studies have been conducted to define the neurophysiological processes that act on sheep as determined by photoperiod (reviewed by Gerlach and Aurich, 2000) and the increases in testicular size, spermatogenesis and semen quality that occur in rams during the time of short day-length (Mickelson *et al.,* 1981; Gerlach and Aurlich, 2000).

In northern and central Queensland, Merino sheep will breed throughout the year although graziers normally breed their sheep in autumn (April-May) or spring / early summer (August-November). Frequently, the exact time of the year when sheep are bred is dictated by the availability of quality pastures, weather and rainfall forecasts as well as other management issues. However, in this tropical environment, there appears to be no published information on changes in semen quality and motility of spermatozoa at different times of the year. This Chapter reports on studies to examine the changes in semen and spermatozoal motility parameters over a 13 month period and the variation in these parameters within and between Merino rams.

5.2 Materials and Methods

5.2.1 Climatic conditions

Data on climatic conditions were obtained from the Commonwealth Bureau of Meteorology Townsville station (altitude: 9^{o} 14' 52" S; longitude: 146^{o} 46' 01" E; elevation: 7.5 m) the area in which the sheep were located. Table 5.1 lists the mean total bright sunshine (hours), day-length (hours), minimum and maximum temperature (^{o}C), precipitation (mm), evaporation (mm) and relative humidity (%) for the study period (June 2002 – July 2003).

5.2.2 Animals

Six Merino rams (eartag numbers R5, R9, R10, R12, R13 and R16) of proven fertility were available for use in this study. The rams were at the following ages at the commencement of the study: R5-4.5 years; R9-1.75 years; R10-3.0 years; R12-1.75 years; R13-1.5 years; R16-1.5 years. Ram semen was collected at approximately four week intervals from June 2002 through to July 2003, using an electroejaculator (see Section 3.4). The rams were not permitted to mate naturally during this period.

5.2.3 Semen analysis

The volume, colour and concentration of spermatozoa were determined as described in section 3.5. The motility, velocity and morphological parameters of spermatozoa were determined with a CASA (see Section 3.6).

5.2.4 Statistical analyses

All data were analyzed using SPSS software program (SPSS 11.0 Brief Guide, New Jersey, USA). Univariate analysis of variance was used to determine the effect of rams and months on the semen characteristics. Analysis of variance one way classification was used to determine the effect of climatic conditions (bright sunshine, day-length, temperature, maximum air temperature, precipitation,

evaporation, and humidity) on the motility, velocity and morphology of sperm head characteristics. The level of significance was considered to be $P \leq 0.05$. Bivariate correlations were used to determine the correlation between season (bright sunshine, day-length temperature, maximum air temperature, precipitation, evaporation, and humidity) and semen quantity and quality.

Table 5.1 Mean daily climatic data for the period June 2002 – July 2003.

Month	Bright sunshine* (hours)	Day-length** (hours)	Min temperature (°C)***	Max temperature (°C)***	Precipitation (mm)	Evaporation (mm)	Relative humidity (%)
June	8.40	11.06	8.3	26.2	0.6	5.3	62.4
July	8.80	11.30	6.3	27.7	0.0	4.8	65.4
August	9.20	11.85	11.1	28.8	0.3	5.5	70.2
September	10.60	12.35	12.1	30.6	0.0	7.0	67.4
October	10.80	12.88	16.4	35.7	0.0	8.6	65.3
November	11.70	13.10	18.8	32.0	0.0	10.6	62.2
December	10.70	13.10	21.9	35.9	0.8	9.8	66.9
January	8.90	13.06	24.3	34.4	0.6	9.8	64.4
February	8.10	12.46	22.9	32.0	11.3	8.2	76.7
March	8.60	12.07	21.0	31.6	2.4	7.5	70.1
April	8.90	11.39	17.5	30.5	0.7	6.6	69.7
May	7.90	11.13	13.8	28.8	0.3	5.7	68.1
June	8.0	10.72	11.4	27.4	0.2	4.8	71.3
July	9.3	11.04	9.9	26.8	0.0	6.4	61.5
CV (%)	12.9	7.2	37.7	10.4	24.1	27.1	6.2

*Bright sunshine is solar radiation intense enough to cast distinct shadows. **Day-length is the time from sunrise to sunset. ***Min = minimum air temperature, Max = maximum air temperature.

5.3 Results

5.3.1 Effect of ram and month on the semen characteristics

Semen volume, semen colour, motility and concentration of spermatozoa of Merino rams varied between rams (Table 5.2). The average semen volume and semen colour were 0.77 ± 0.03 ml with a range of 0.2 ml to 1.60 ml and 2.6 ± 0.16 with a range of 0.0 to 5.0 ml, respectively. The average motility and concentration of spermatozoa

was 66.3 ± 2.6% with a range of 0.0% to 90% and 2044 ± 164 million/ml with a range of 10 million/ml to 6210 million/ml, respectively. The ram with the highest mean semen volume was R10 and the ram with the lowest was R5. The ram with the best semen colour, motility and concentration of spermatozoa was R9 and R16 and R12 had the lowest sperm motility and sperm concentration, respectively.

Table 5.2 Mean (± SEM) values of the semen characteristics for each ram during the period of study.

Ram number	Semen volume (ml)	Semen colour (consistency)	Sperm motility (%)	Sperm concentration (x 10^6 / ml)
5	0.61 ± 0.08[a]	3.0 ± 0.43[a]	70.4 ± 6.8[a]	2551 ± 551[a]
9	0.85 ± 0.04[b]	3.7 ± 0.24[b]	81.5 ± 2.0[b]	3089 ± 324[a]
10	0.99 ± 0.08[c]	2.8 ± 0.37[a]	65.4 ± 6.9[a]	2045 ± 435[b]
12	0.80 ± 0.07[b]	2.1 ± 0.37[c]	63.5 ± 5.7[c]	1521 ± 280[c]
13	0.65 ± 0.06[a]	2.1 ± 0.33[c]	60.6 ± 6.9[c]	1537 ± 306[c]
16	0.70 ± 0.05[a]	2.1 ± 0.40[c]	56.9 ± 6.8[c]	1564 ± 336[c]
Overall	0.77 ± 0.03	2.6 ± 0.16	66.3 ± 2.6	2044 ± 164
Range	0.2 - 1.60	0.0 - 5.0	0.0 - 90.0	10 - 6210

Data with different superscripts within each column were significantly different ($P \leq 0.05$).

The semen characteristics were affected by month although there was no effect of months on the semen volume, semen colour, motility and concentration of spermatozoa varied between months (Table 5.3). The best semen colour (creamy) was in July and the poorest (clear) was in March. The lowest motility and concentration of spermatozoa was also in March whereas the best motility was in October (94.83%) and the best concentration was in November (3773 million/ml).

Table 5.3 The mean (± SEM) values for semen characteristics of the six rams for each month during the study.

Month	Semen volume (ml)	Semen colour (consistency)	Sperm motility (%)	Sperm concentration (x 10^6 / ml)
June	0.73 ± 0.06	3.1 ± 0.26^{b}	81.05 ± 12.3^{a}	1640 ± 180^{ab}
July	0.78 ± 0.06	3.7 ± 0.21^{b}	88.67 ± 5.6^{b}	2950 ± 262^{bc}
August	0.73 ± 0.09	3.2 ± 0.48^{b}	90.50 ± 7.9^{b}	1988 ± 359^{ab}
September	0.73 ± 0.09	2.5 ± 0.50^{ab}	92.00 ± 2.6^{b}	2017 ± 416^{ab}
October	0.69 ± 0.10	3.0 ± 0.51^{b}	94.83 ± 2.4^{b}	2261 ± 430^{ac}
November	0.82 ± 0.09	3.3 ± 0.61^{b}	79.12 ± 9.8^{b}	3773 ± 702^{bc}
December	0.84 ± 0.08	2.7 ± 0.42^{ab}	83.50 ± 8.1^{b}	2377 ± 277^{ac}
January	0.69 ± 0.09	1.5 ± 0.56^{a}	55.33 ± 12.5^{ac}	903 ± 413^{a}
February	0.85 ± 0.13	2.3 ± 0.42^{ab}	78.00 ± 8.2^{a}	1694 ± 551^{ab}
March	0.76 ± 0.19	0.8 ± 0.31^{a}	39.00 ± 18.5^{c}	315 ± 193^{a}
April	0.89 ± 0.10	2.7 ± 0.62^{ab}	70.33 ± 9.5^{a}	2627 ± 723^{bc}
May	0.68 ± 0.12	2.4 ± 0.61^{ab}	77.50 ± 6.65^{a}	1810 ± 592^{ab}
June	0.66 ± 0.05	3.0 ± 0.41^{b}	72.30 ± 3.3^{a}	2298 ± 476^{ac}
July	0.69 ± 0.08	3.2 ± 0.42^{b}	60.17 ± 8.8^{b}	2664 ± 492^{bc}
Overall	0.75 ± 0.07	2.67 ± 0.73	75.64 ± 14.6	2094 ± 817
Range	0.20 - 1.60	0.0 - 5.0	0.00 - 100.0	10 - 6120

Data with different superscripts within each column were significantly different ($P \leq 0.05$).

The coefficient of variation between and within rams in semen characteristics varied. The coefficient of variation of semen volume within rams was better than between rams, being 9.7% and 18.4% respectively whereas the coefficient of variation of sperm motility between rams was better than within rams (Table 5.4). However the coefficient of variation of semen colour and sperm concentration between and within rams was relatively high.

Table 5.4 Coefficiant of variation (%) between and within rams in semen characteristics.

Parameters	Semen volume	Semen colour	Sperm motility	Sperm concentration
Between ram	18.4	25.0	13.1	31.7
Within ram	9.7	28.5	20.0	40.5

5.3.2 Effect of month on the motility characteristics of spermatozoa

Time of the year significantly affected the percentage of motile, progressively motile and rapidly motile spermatozoa. In January and March the percentage of motile, progressively motile and rapidly motile spermatozoa were significantly decreased compared to other months (Figure 5.1). The percentage of motile, progressively motile and rapidly motile spermatozoa was significantly increased in June through to October (81% - 95%, 38% - 67%, 52% - 80%, respectively). The percentage of medium and slow motile spermatozoa remained relatively constant throughout the year at about 10-15%.

Although VAP, VSL and VCL varied slightly between months it was in March that VAP, VSL and VCL were significantly decreased (Figure 5.2). Straightness (STR), linearity (LIN) and elongation (ELO) of spermatozoa varied slightly between months but it was in March that these parameters were decreased significantly (Figure 5.3). The amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and head area of spermatozoa fluctuated slightly between months but there was a significant decrease in these parameters in March (Figure 5.4).

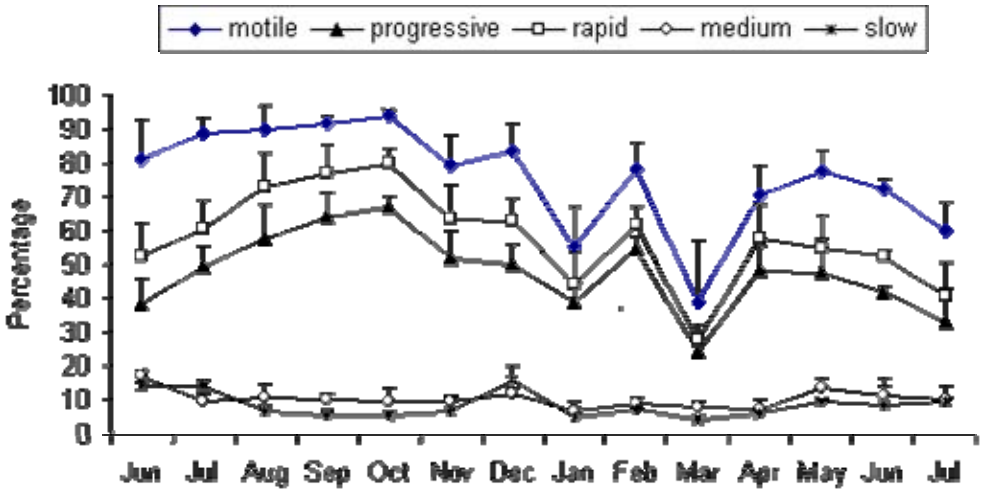


Figure 5.1 The effect of months on the percentage of motile and progressive, rapid, medium and slowly motile ram spermatozoa. The results are the mean (± SEM) of the data from six rams.

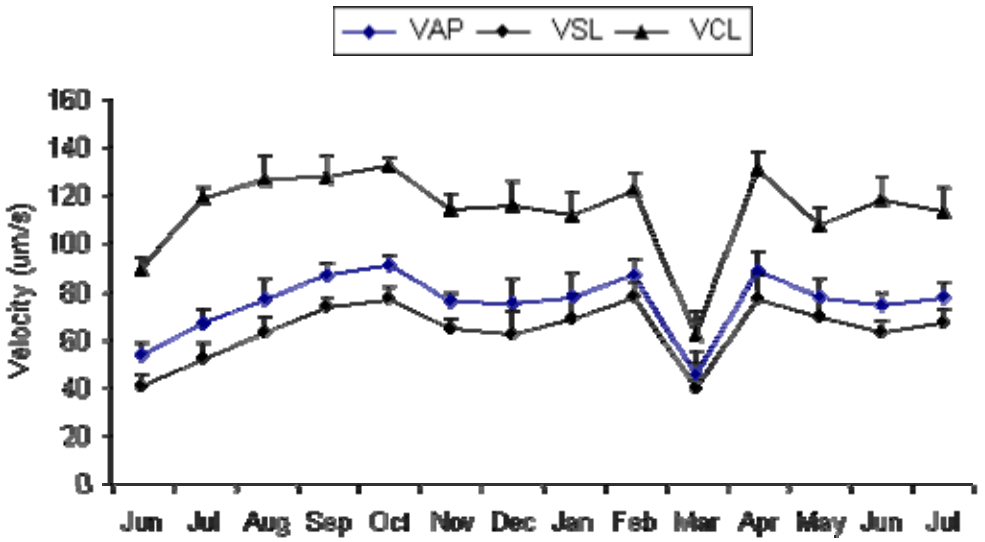


Figure 5.2 Effect of months on the average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) of ram spermatozoa The results are the mean (± SEM) of the data from six rams.

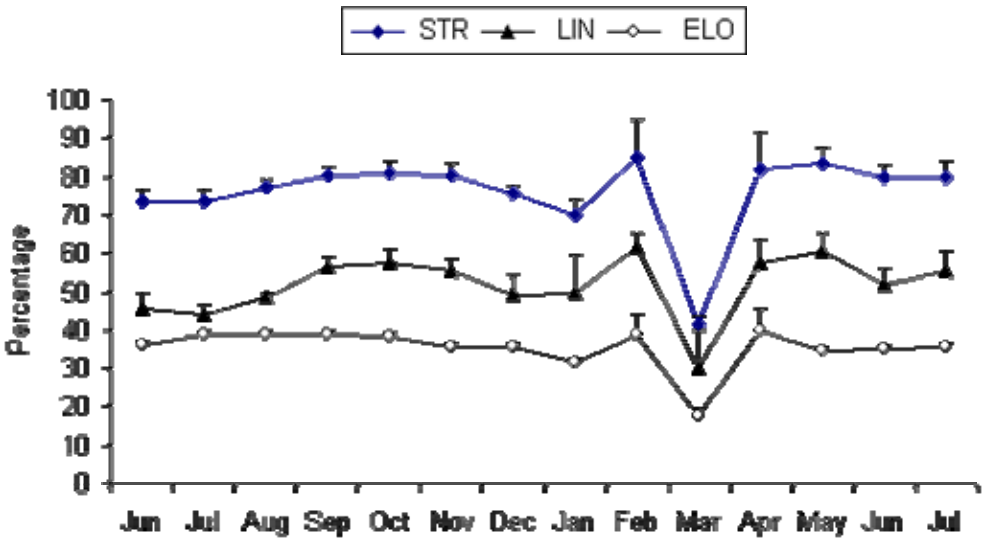


Figure 5.3 Effect of months on the straightness (STR), linearity (LIN) and elongation (ELO) of ram spermatozoa. The results are the mean (± SEM) of data from six rams.

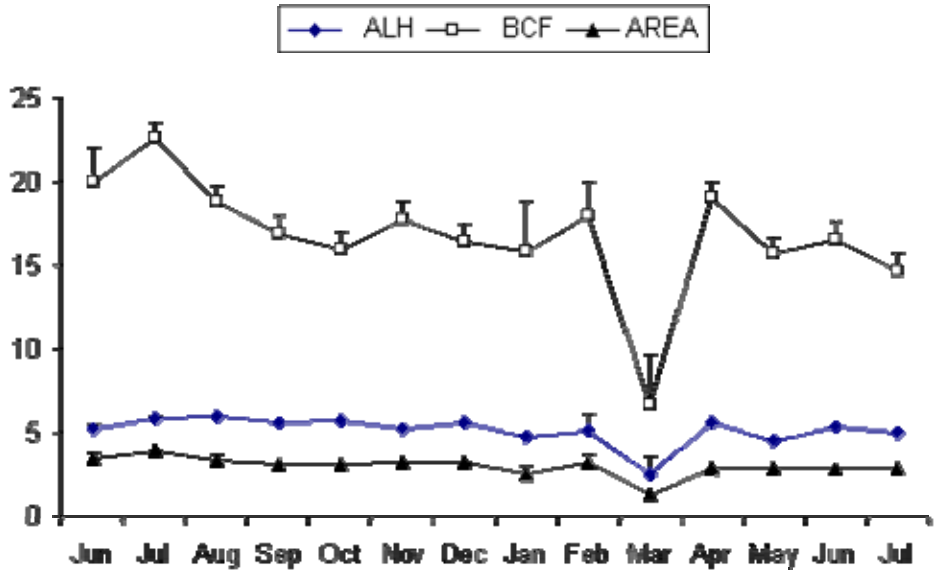


Figure 5.4 Effect of months on the amplitude of lateral head displacement (ALH) beat cross frequency (BCF) and head area (AREA) of ram spermatozoa. The results are the mean (± SEM) of data from six rams.

5.3.3 Effect of ram on the motility and morphology characteristics of spermatozoa

The mean percentage of motile, progressive, rapid, medium and slow motile spermatozoa varied between rams (Figure 5.5 A). R9 had the highest results with a mean value of 94.2% for motile, 58.1% for progressive and 74.9% for rapid spermatozoa. This ram also had the highest VCL (125.9 µm/s) compared with the lowest VCL of 103.5 µm/s from R10 (Figure 5.5 B). Straightness, LIN and ELO (Figure 5.5 C) were not significantly different between rams. Elongation and head area of spermatozoa were not significantly different between rams (Table 5.5).

Table 5.5 Effect of ram (mean ± SEM) on the elongation and head area of spermatozoa.

Rams	Elongation (%)*	Head area (μm^2)*
R5	35.05 ± 3.4	2.95 ± 0.28
R9	38.75 ± 1.1	3.43 ± 0.24
R10	35.42 ± 3.3	2.80 ± 0.29
R12	36.42 ± 4.4	3.28 ± 0.48
R13	33.67 ± 3.2	2.82 ± 0.33
R16	33.00 ± 3.1	2.76 ± 0.26

* There was no significant difference between rams

The percentage of motile, progressively motile and rapidly motile spermatozoa varied considerably within each ram and showed a high coefficient of variation, except for R9 where the coefficient of variation was 8.0% for motile, 19.7% for progressively motile and 21.1% for rapidly motile spermatozoa (Table 5.6).

5.3.4 Correlation (r) between climatic conditions and semen characteristics of rams

The motility and concentration of spermatozoa was significantly correlated with the hours of bright sunshine (Table 5.7). Day-length, maximum air temperature, precipitation, evaporation and relative humidity were not significantly correlated

with semen volume, semen colour/consistency, motility and concentration of spermatozoa.

There was a significant positive correlation between the maximum air temperature and the percentage of motile spermatozoa and beat cross frequency (Table 5.8). The hours of bright sunshine significantly affected the percentage of progressively motile and rapidly motile spermatozoa. Day length, precipitation, evaporation and humidity did not significantly affect the percentage of motile spermatozoa, the percentage of progressively motile and rapidly motile spermatozoa or the velocity characteristics of spermatozoa.

Table 5.6 The percentage of motile (M), progressively motile (P) and rapidly motile (R) spermatozoa of each ram and their coefficient of variation (CV).

Month	Ram 5			Ram 9			Ram 10			Ram 12			Ram 13			Ram 16		
	M	**P**	**R**	**M**	**P**	**R**	**M**	**P**	**R**	**M**	**P**	**R**	**M**	**P**	**R**	**M**	**P**	**R**
Jun	87	38	69	98	67	76	92	39	56	95	48	65	20	4	8	94	32	41
Jul	97	73	88	87	49	49	92	46	68	96	27	49	98	53	58	62	49	53
Aug	99	69	93	99	74	92	98	37	49	100	66	89	51	40	41	96	59	73
Sep	95	67	80	93	66	83	91	51	58	99	74	96	94	62	79	80	64	67
Oct	98	72	91	99	72	92	97	74	87	95	52	69	83	55	59	97	76	82
Nov	99	76	95	99	56	81	50	38	43	50	29	31	99	70	84	78	42	46
Dec	95	81	90	96	50	83	99	71	90	51	12	17	93	75	82	67	11	17
Jan	92	62	77	71	37	45	60	50	51	51	31	38	0	0	0	58	52	55
Feb	97	78	85	96	66	88	67	53	57	75	39	42	88	56	59	45	38	41
Mar	0	0	0	98	60	72	0	0	0	85	39	44	51	45	49	0	0	0
Apr	82	32	47	98	60	85	72	38	49	31	29	31	81	75	77	58	56	56
May	79	48	53	96	40	53	68	39	43	52	45	45	77	60	63	93	53	71
Jun	97	62	83	97	58	76	97	47	66	12	11	11	42	34	35	89	37	43
Jul	14	11	11	99	72	93	68	50	52	35	8	11	59	32	37	86	24	38
CV (%)	**39**	**46.6**	**44.3**	**7.97**	**19.7**	**21.1**	**36.1**	**38.5**	**39**	**44.5**	**54.4**	**57.9**	**45.9**	**49.8**	**49.9**	**36.9**	**48.8**	**45.1**

0 – semen collection unsuccessful

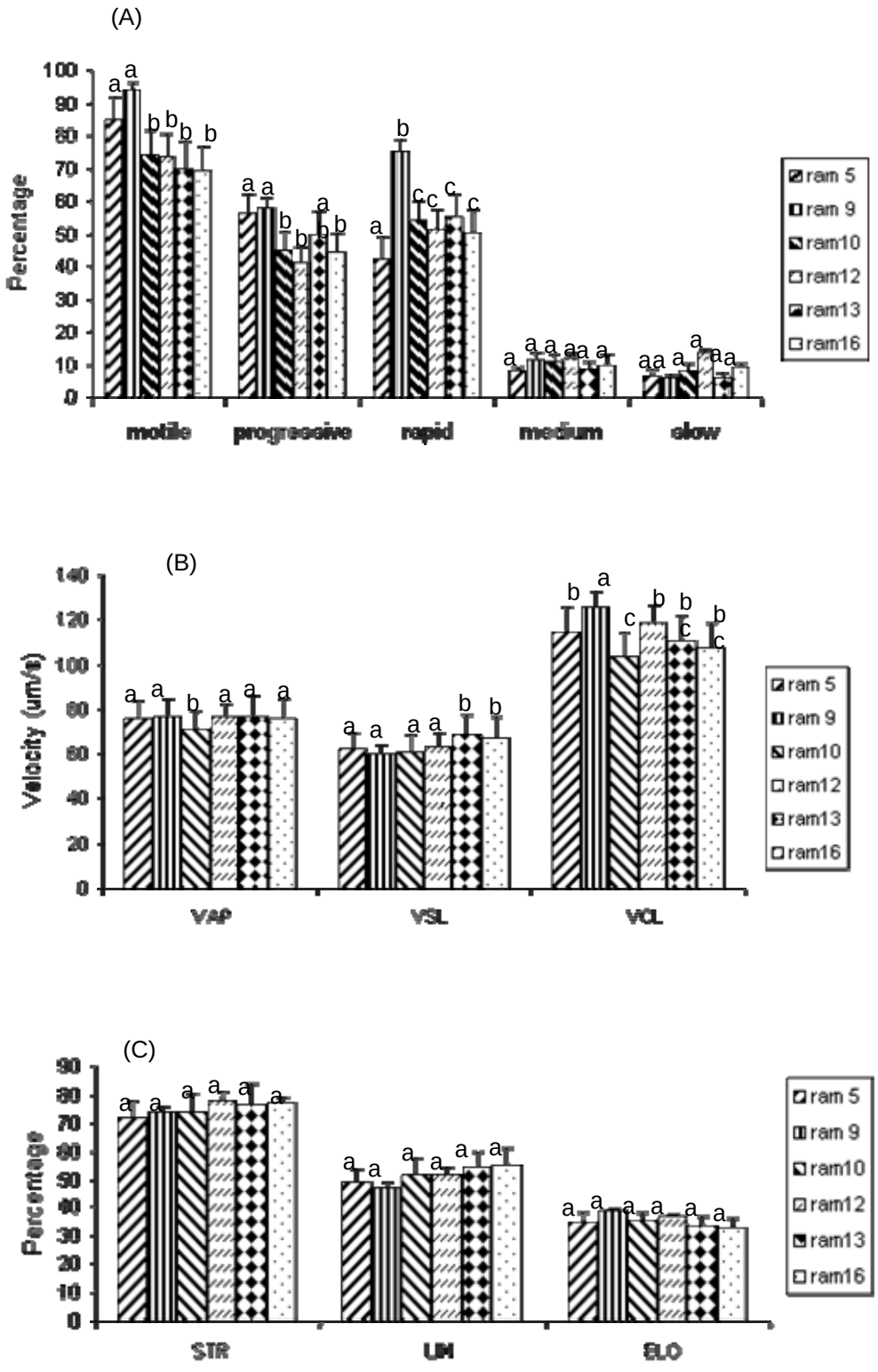


Figure 5.5 Effect of rams on the percentage of motile and progressive, rapid, medium and slowly motile spermatozoa (Figure A), average path velocity(VAP), straight-line velocity (VSL), and curvilinear velocity (VCL) (Figure B), straightness (STR), linearity (LIN) and elongation (ELO) (Figure C) of spermatozoa. The results are the mean (± SEM) of 14 replicates for each ram. Different letters above bars indicate significant differences within each group

Table 5.7 Correlation (r) between climatic conditions and semen characteristics of rams.

Parameters	Semen volume	Semen colour (consistency)	Sperm motility	Sperm concentration
Day-length	0.29	-0.32	-0.07	-0.06
Maximum air temperature	0.09	-0.35	-0.16	-0.24
Bright sunshine	0.21	0.26	0.49*	0.51*
Precipitation	0.42	-0.31	-0.34	-0.28
Evaporation	0.34	-0.33	-0.13	0.04
Humidity	0.28	-0.33	-0.29	-0.34

* Significant ($P \leq 0.05$).

Table 5.8 Correlation (r) between climatic conditions and motility characteristics of ram spermatozoa.

Parameters	Mot	Prog	Rap	VAP	VSL	VCL	ALH	BCF	STR	LIN
Day-length	0.21	-0.11	-0.03	-0.31	-0.37	-0.13	0.08	0.35	0.02	-0.15
Maximum temperature	0.52*	-0.17	-0.29	-0.22	0.35	-0.08	0.39	0.55*	-0.14	0.13
Bright sunshine	0.33	0.44*	0.47*	0.29	0.24	0.31	0.30	0.01	0.19	0.18
Precipitation	-0.16	-0.12	0.12	0.21	-0.03	-0.18	-0.08	0.07	0.20	0.21
Evaporation	-0.23	0.05	-0.01	0.26	0.33	0.07	-0.11	-0.32	0.01	0.17
Humidity	-0.13	0.08	-0.03	0.24	0.32	0.09	-0.17	-0.20	0.04	0.17

* Significant ($P \leq 0.05$), Mot = motile sperm, Prog = progressively motile, Rap = rapidly motile, VAP = average path velocity, VSL = straight-line velocity, VCL = curvilinear velocity, ALH = amplitude of lateral head displacement, BCF = beat cross frequency, STR = straightness, LIN = linearity.

5.4 Discussion

There studies have highlighted the variation in semen parameters of rams during a 13 month period. The period January to March was the time when the overall semen quality was poorest as measured by the mean concentration of spermatozoa, percentage motility and detailed velocity measurements. January to March is the time of the year in this environment when there is a combination of high

temperatures and high humidity. However, the combined effect of high temperature and high humidity seemed to vary considerably between rams. Ejaculates from R9 contained spermatozoa of good motility during January to March whereas the other five rams showed variable results from no spermatozoa present in the ejaculate to ejaculates containing spermatozoa of good motility.

It is well recognised that elevated temperatures will affect spermatogenesis. Rams have a well developed cooling system in the testis and scrotum that enable the intra-testicular temperature to be about 4.5 ^{o}C lower than core body temperature (Setchell, 1991). The cooling processes of the scrotum can be affected by a number of factors such as excessive scrotum fat, dermatitis of the scrotal skin or excess dirty and matted wool. The rams in this study had three months growth of wool at the beginning of January. High humidity would have reduced the heat exchange from the scrotum. Given that spermatogenesis in the ram takes seven weeks (Johnson and Everitt, 2000), the detrimental effect of high temperature and high humidity would have commenced during late November and early December.

Semen of best quality was produced during the period July to December. This corresponds to the period of increasing daylength and is also one of the two preferred times of the year for graziers in north Queensland to breed Merino sheep. This is opposite to the situation in temperate Australia where rams and ewes are influenced by photoperiod and have maximum fertility during autumn, although nutrition will also have a significant role at least in Merino rams (Lindsay, 1991; Martin *et al.,* 2002).

There are a limited number of reports on the quality of ram semen in tropical environments. Joshi *et al.* (2003) studied the motility characteristics of Garole rams in a semi-arid tropical environment with CASA. Their study was confined to semen collected for three weeks each autumn for three years and the authors concluded that Garole rams could produce good quality semen after prolonged exposure to a semi-arid tropical climate. Ibrahim (1997) studied local and crossbred rams in the United Arab Emirates and concluded that high ambient temperatures did not affect the sexual activity of rams although the best quality semen as determined by volume, concentration of spermatozoa and motility was produced during the winter months.

In Chapter 4, a significant difference between rams in the head area of spermatozoa was identified. At initial incubation in TALP medium, R12 had a significantly greater spermatozoa head area than R9, R13 and R16 (Figure 4.C). In the study in this Chapter, a significant difference between rams did not occur although it was R12 that had the greatest standard error for this measurement. The reason for this variation in results was not apparent although consistent with the results in Chapter 4, R13 and R16 had a similar head area and R9 had spermatozoa with a mean head area that was greater than R13 and R16.

The rams did not respond adversely to repeated electroejaculation at monthly intervals although on a couple of occasions a ram did not respond to electroejaculation during the period January to March. A repeated attempt five to seven days later was successful. Lambrechts *et al.* (2000) collected semen by electroejaculation from Merino rams weekly for eight weeks and did not report any difficulties. The technique of electroejaculation in rams has been used for many years (Cameron, 1977) but it is recognised the Institutional Animal Ethics Committees in some states of Australia and some other countries do not approve of the technique. Collection by artificial vagina is the preferred technique although the rams used in this study had not been trained for this procedure.

In conclusion, this Chapter provides data on the influence of climatic conditions and variation between rams on the parameters of semen collected by electroejaculation from Merino rams in a tropical environment. The hot summer months of January to March had a detrimental effect on the quality of semen most likely due to elevated intra-testicular temperatures and subsequent interference with spermatogenesis.

CHAPTER 6

TEMPORAL CHANGES IN MOTILITY CHARACTERISTICS, RECOVERY AND DIMENSIONS OF SPERMATOZOA AT VARIOUS SITES IN THE REPRODUCTIVE TRACT OF MERINO EWES

6.1 Introduction

The literature contains a number of reports on the distribution of spermatozoa within the female reproductive of mammals following natural or artificial insemination. These results include work on sheep (Schott and Phillips, 1941; Quinlivan and Robinson, 1969; Mattner and Braden, 1969a, 1969b; Allison and Robinson, 1970; Lightfoot and Salamon, 1970; Hawk and Conley, 1971; Allison, 1972; Hawk and Cooper, 1975; Hawk and Cooper, 1977; Hawk *et al*., 1978; Hawk and Cooper, 1984; Lane *et al*., 1993), goats (Cox *et al*., 2002), pigs (First *et al*., 1968; Kaeoket *et al*., 2002; Langendijk *et al*., 2002), and cattle (Hawk, 1987). In some of the studies, the influence of factors such as progestagen treatment for oestrus synchronisation, stage of oestrus at mating and the motility of spermatozoa on the distribution of spermatozoa were studied.

Two prominent features have emerged from the above studies. One is that only a small percentage of spermatozoa that were inseminated can be recovered from the female reproductive tract and the second is that there is a substantial reduction in the number of spermatozoa as one moves anteriorly along the female reproductive tract. For example, in sheep Mattner and Braden (1969b) recovered 17.22 x 10^6 spermatozoa from the cervix, 101,000 from the uterus and only 13 from the oviducts four hours after deposition of 550 x 10^6 spermatozoa within the first fold of the cervix. In the pig, a species in which there is intra-uterine insemination in natural mating, Langendjk *et al.* (2002) recovered 331,000 spermatozoa from the uterine horns, a similar number from the utero-tubal junction and about 500 from the oviducts 12 hours after insemination with 3 x 10^9 spermatozoa.

The biological features of both spermatozoa and the female reproductive tract that determine the distribution of spermatozoa leading to a population of spermatozoa in the oviducts capable of fertilisation are not known. The experiments reported in this

Chapter were undertaken to test the hypothesis that the motility, velocity, and morphological features of spermatozoa that had progressed to the anterior uterus and oviducts were different to those in the posterior reproductive tract. A further aim was to determine the effect of time after mating on the recovery of spermatozoa from the female reproductive tract of Merino ewes.

6.2 Materials and Methods

6.2.1 Animals

Four rams (eartag numbers R9, R12, R13, R16) aged 1.5 years at the commencement of the study were used for mating ewes. The number of ewes that were used to collect spermatozoa at different sites in the reproductive tract is presented in Table 6.1. The management of sheep and the methods for oestrus and mating control have been described in Section 3.1 and 3.7 respectively. After mating, samples of spermatozoa were collected from the female reproductive tract of ewes as described in Section 3.8.

Table 6.1 The number of ewes from which samples of spermatozoa were collected from different sites of the reproductive tract.

Time after mating (hours)	Number of ewes			
	Anterior vagina *	Cervix	Uterus	Oviducts
1	20	0	0	0
3	23	8	8	0
6	20	14	14	8
24	13	13	13	9

*Nine ewes had samples collected from the anterior vagina at 1 and 3 hours, 7 ewes at 1, 3 and 6 hours, 3 ewes at 1, 3, 6 and 24 hours, one ewe at 1, 3 and 24 hours, two ewes at 3 and 6 hours, one ewe at 3 hours, 8 ewes at 6 hours and 9 ewes at 24 hours after mating.

6.2.2 Reaction time, mating and frequency of mating

The reaction time, number of matings and mating frequency of rams during the one hour mating period are summarized in Table 6.2. The reaction time was defined as time for the ram to show interest in the ewe and a mating wass defined as when intromission had occurred. The mean reaction time was 13.8 ± 5.5 minutes and the mean number of matings and mating frequency was 2.8 ± 0.2 times and 12.1 ± 1.1 minutes, respectively.

Table 6.2 The performance of Merino rams during the one hour mating period (mean ± SEM).

Ram No.	Number of times rams were used	Reaction time (minutes)	Number of matings	Frequency of mating (minutes)
9	10	15.8 ± 5.6 [a]	2.7 ± 0.9 [a]	13.5 ± 6.5 [a]
12	10	4.9 ± 4.4 [b]	3.0 ± 1.6 [a]	11.2 ± 4.5 [a]
13	9	20.0 ± 12.7 [a]	2.5 ± 0.9 [a]	12.8 ± 6.1 [a]
16	9	14.3 ± 8.5 [a]	3.0 ± 2.4 [a]	11.0 ± 4.0 [a]
Overall		13.8 ± 5.5	2.8 ± 0.2	12.1 ± 1.1
Range		1 - 47	1 - 7	5 - 25

Data with different superscripts within each column were significantly different ($P \leq 0.05$).

6.2.3 Determination of motility characteristics and number of spermatozoa in the female reproductive tract

Determination of motility characteristics and the number of spermatozoa in the female reproductive tract of ewes after mating have been described in Sections 3.8.2 and 3.8.4 respectively.

6.2.4 Measurement of the size of spermatozoa

Smears of the samples from the reproductive tract of ewes were made on microscope slides and stained with nigrosin-eosin. Smears were also made from six rams (eartag numbers R5, R9, R10, R12, R13 and R16) following semen collection for studies described in Chapter 4. Those spermatozoa that were lying in a straight-line were

measured for total length, length of tail and length and width of the head using an objective micrometer (one unit = 2.6 μm).

6.2.5 Statistical analyses

All data were analysed using SPSS software program (SPSS 11.0 Brief Guide, New Jersey, USA). Analysis of variance (ANOVA) one way classification was used to determine the effect of time after mating on the recovery of spermatozoa, motility, velocity, morphology and size of spermatozoa. The differences between means were tested by the Least Significant Difference test. Furthermore, the Student t-test was used to determine the effect of the ipsilateral and contralateral side on the number of spermatozoa recovered from the uterine horns and oviducts. The level of significance was considered to be $P \leq 0.05$.

6.3 Results

6.3.1 Recovery of spermatozoa from the reproductive tract of ewes

The concentration of spermatozoa in the samples collected from the anterior vagina decreased significantly with time after mating as was the case with number of spermatozoa recovered from the cervix, uterus and oviducts (Figure 6.1). At 1, 3, 6 and 24 hours after mating the mean (± SEM) number of spermatozoa collected in the samples taken from the anterior vagina was 2493 ± 1298 x 10^6, 1990 ± 854 x 10^6, 593 ± 252 x 10^6 and 21.9 ± 9.8 x 10^6 respectively (Figure 6.1A).

A mean of more than 20,000 spermatozoa were recovered from the posterior and midcervix three hours after mating and significantly fewer from the anterior cervix (Figure 6.1 B). By 6 hours, there was significant reduction in number of spermatozoa in all sites of the cervix. The number of spermatozoa recovered from midcervix and anterior cervix was similar (about 5,000) at 6 and 24 hours after mating although fewer spermatozoa were recovered from the posterior cervix at 24 hours than at 6 hours. At 6 hours, the mean number of spermatozoa recovered from the right isthmus, right ampulla, left isthmus and left ampulla was 400 ± 25, 140 ± 15, 90 ± 5 and 120 ± 25 respectively and at 24 hours the recovery was 200 ± 50, 130 ± 25, 150 ± 30 and 100 ± 15, respectively (Figure 6.1 B). The number of spermatozoa recovered from the mid and anterior uterine horn at 3 hours after mating was 4040 ±

180 and 2850 ± 525, respectively and by 24 hours this had declined to 1590 ± 70 and 930 ± 178, respectively.

The number of spermatozoa recovered was influenced by the sites of the reproductive tract of ewes. For example, at 6 hours after mating the number of spermatozoa recovered from the cervix was significantly higher ($P \leq 0.05$) than the number recovered from the uterus and oviduct. At 24 hours after mating, the number of spermatozoa recovered from the cervix was significantly higher (about 5000 spermatozoa) than the uterus and oviduct (Figure 6.1 B).

6.3.2 Effect of the side of the preovulatory follicle or corpus haemorrhagicum on the number of spermatozoa recovered from the uterine horns and oviducts

The effect of the side of the preovulatory follicle or corpus haemorrhagicum on the number of spermatozoa recovered from the uterine horns and oviducts was analysed from 10 ewes where there was an accurate determination of the number of spermatozoa in the samples (Table 6.3). A consistent finding was that there were always significantly more ($P \leq 0.05$) spermatozoa in the oviduct ipsilateral to the preovulatory follicle or corpus haemorrhagicum. Significantly more spermatozoa were present in the ipsilateral mid- and anterior uterine horn 24 hours after mating and a similar trend, although not significant, was present in the anterior uterus 6 hours after mating. The average recovery of spermatozoa from the oviducts of ewes 24 hours after mating was more than at 6 hours after mating in both the ipsilateral and contralateral side, but the reverse was the case with the recovery from the uterine horn (Table 6.3).

6.3.3 Motility of spermatozoa recovered from the reproductive tract of ewes

The mean percentage of motile, progressively motile and rapidly motile spermatozoa in the anterior vagina at 1 hour after mating was 58.8%, 30.6% and 41.2% decreasing significantly to 16.2%, 7.3% and 9.2% motile spermatozoa at 3 hours, 5.2%, 1.5% and 1.9% at 6 hours and 1.9%, 0.5% and 1% at 24 hours after mating, respectively (Figure 6.2). The decline in the percentage of motile spermatozoa in the cervix was not as marked as in the vagina with about 20 – 30% being motile at 3 and 6 hours after mating declining to about 13% by 24 hours. About 15% of the

spermatozoa in the uterine body were motile at 3 and 6 hours declining to 1.2% by 24 hours. Elsewhere the percentage of motile spermatozoa in the uterine horns was variable at 3 and 6 hours after mating (range 2% – 22%) with about 2.3% (range 1.7% - 2.6%) motile spermatozoa in the oviduct. Few spermatozoa (range 1.2% - 6.9%) and (range 2% – 4.5%) were motile in the uterus and oviduct at 24 hours, respectively.

At 3 hours after mating, the percentage of motile, progressively motile and rapidly motile spermatozoa was significantly different between the vagina, cervix and uterus. At 6 hours after mating the percentage of motile spermatozoa in the posterior cervix, body of uterus and mid-uterus was about 15% to 30% but in the other sites was no more than 5%. At 24 hours after mating the percentage of motile, progressively motile and rapidly motile spermatozoa in all the sites of the reproductive tract was very low and was not significantly different (Figure 6.2).

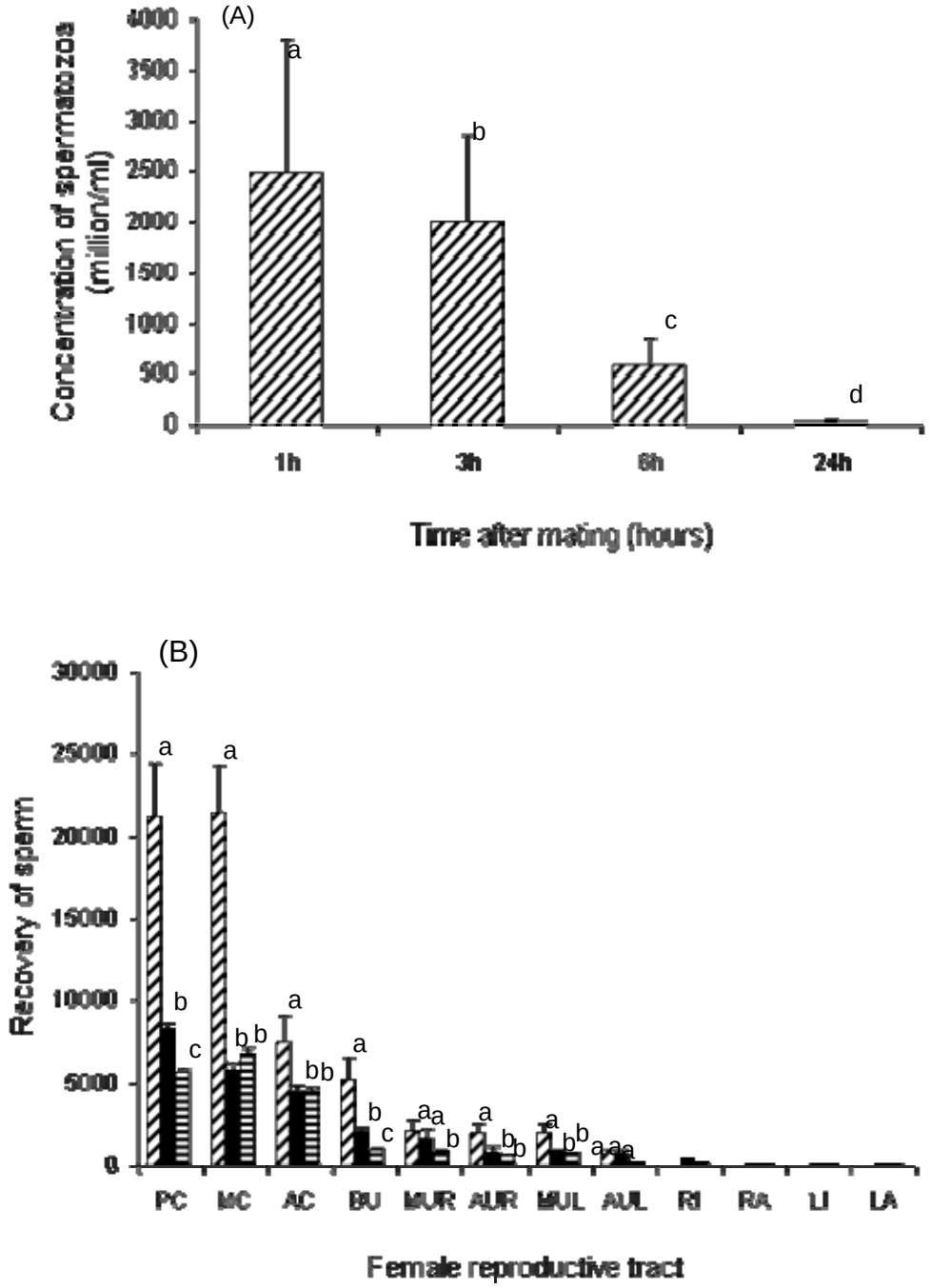


Figure 6.1 The mean (± SEM) concentration of spermatozoa from the anterior vagina (Figure A), of ewes at 1, 3, 6 and 24 hours after mating and the number of spermatozoa recovered from the cervix, uterus and oviducts (Figure B) of ewes at 3, 6 and 24 hours after mating. Different letters above bars indicate significant differences ($P \leq 0.05$) between the time after mating (Figure A) and within that part of the female reproductive tract of ewes (Figure B).AV = anterior vagina; PC, MC, AC = posterior, mid, anterior cervix;BU = body of uterus; MUR, AUR = mid, anterior uterus-right; MUL, AUL =mid, anterior uterus-left; RI, LI = right, left isthmus; RA, LA = right, left ampulla.

Table 6.3 The relationship between the recovery (mean ± SEM) of spermatozoa from the oviducts and uterus and the side of the pre-ovulatory follicle or corpus haemorrhagicum.

Parameter	Number of ewes	Ipsilateral	Contralateral
6 hours after mating:			
Mid-uterus	5	176 ± 47	200 ± 56.4
Anterior uterus	5	200 ± 30	188 ± 47.6
Mean for the uterus		188 ± 12	194 ± 6.0
Isthmus	5	23.8 ± 5.4 [a]	9.8 ± 2.1 [b]
Ampulla	5	21.6 ± 6.2 [a]	6.2 ± 1.8 [b]
Mean for the oviduct		22.7 ± 1.1 [a]	8.0 ± 1.8 [b]
24 hours after mating:			
Mid-uterus	5	70.0 ± 21.7 [a]	46.0 ± 12.1 [b]
Anterior uterus	5	52.0 ± 11.6 [a]	36.0 ± 10.3 [b]
Mean for the uterus		61.0 ± 9.0 [a]	41.0 ± 5.0 [b]
Isthmus	5	34.5 ± 9.0 [a]	16.8 ± 5.62 [b]
Ampulla	5	23.8 ± 8.9 [a]	5.6 ± 1.03 [b]
Mean for the oviduct		29.2 ± 5.4 [a]	11.2 ± 5.6 [b]

Data with different superscripts within a row were significantly different ($P \leq 0.05$).

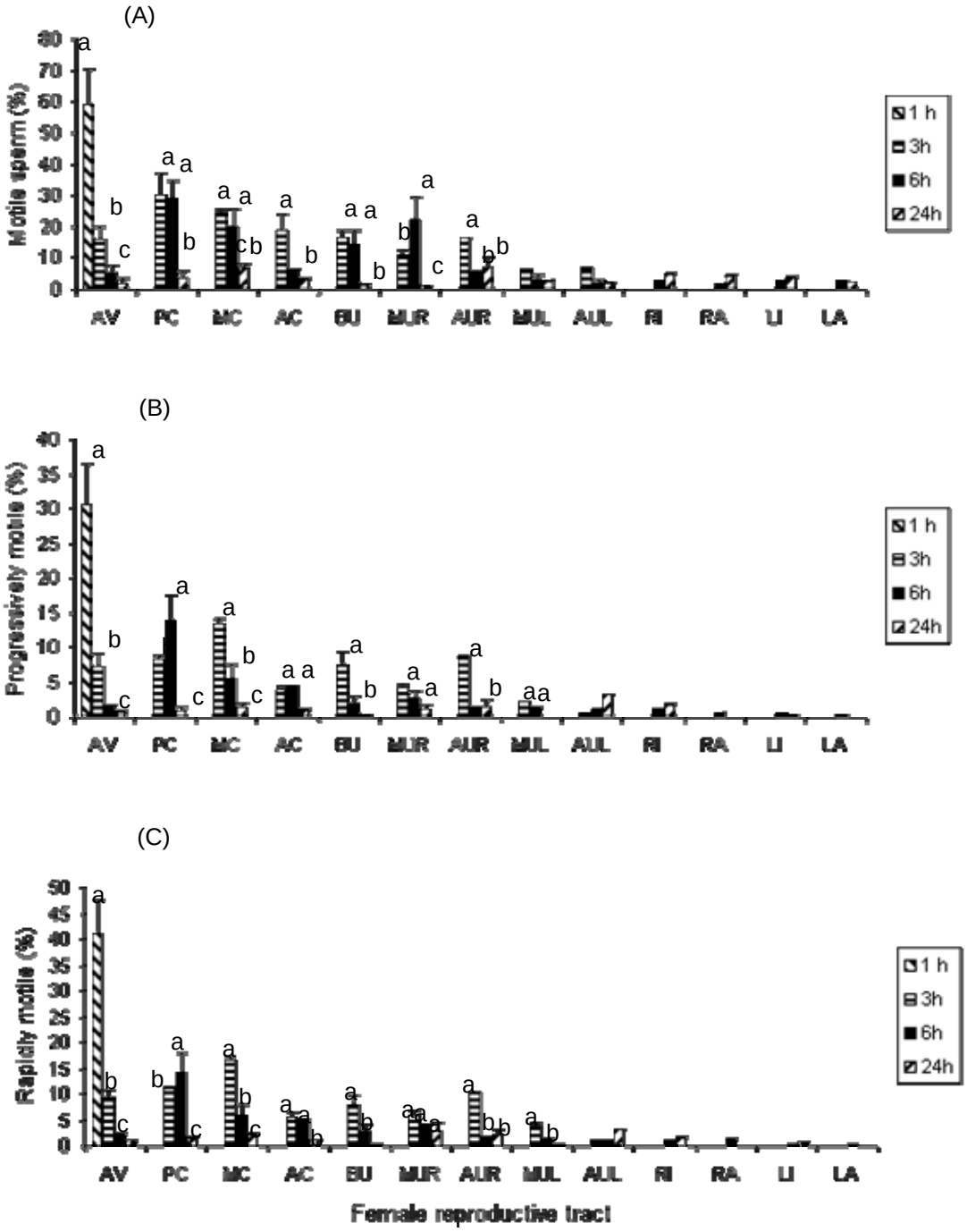


Figure 6.2 The mean (± SEM) percentage of motile (Figure A), progressively motile (Figure B) and rapidly motile (Figure C) spermatozoa in the anterior vagina, cervix, uterus and oviducts of ewes at 1, 3, 6 and 24 hours after mating. Different letters above bars indicate significant differences ($P \leq 0.05$ within that part of the female reproductive tract.
AV = anterior vagina; PC, MC, AC = posterior, mid, anterior cervix; BU = body of uterus; MUR, AUR = mid, anterior uterus-right; MUL, AUL = mid, anterior uterus-left; RI, LI = right, left isthmus; RA, LA = right, left ampulla.

6.3.4 Velocity and morphological features of spermatozoa recovered from the reproductive tract of ewes

Generally the velocity of spermatozoa as defined by VAP, VSL and VCL was significantly better at 6 hours than at 3 or 24 hours after mating in most sites of the reproductive tract of ewes (Figure 6.3) but there were no consistent better velocity characteristics in the anterior reproductive tract compared with the posterior reproductive tract. In fact, the reverse seems to be the case, at least with VAP and VSL where the velocities in the mid- and anterior cervix tended to be greater than at other sites of the reproductive tract.

At 3 hours after mating, VAP, VSL and VCL were significantly different between the vagina, cervix and uterus and in the cervix spermatozoa had a VAP, VSL and VCl that were faster than spermatozoa from the vagina and uterus. At 6 hours and 24 hours after mating the VAP, VSL and VCL of spermatozoa fluctuated between sites of the female reproductive tract. Spermatozoa in the cervix had a significantly greater VAP, VSL and VCL than the other sites (Figure 6.3).

While the data on beat cross frequency, amplitude of lateral head displacement and straightness (Figure 6.4) and linearity, elongation and head area (Figure 6.5) did show some significant differences between times after mating at different sites, the data did not show any consistent trends across the various sites in the reproductive tract. The only exception was the significantly smaller head area and smaller elongation percentage of spermatozoa in the oviducts compared with other sites (Figure 6.5 B,C). The data for head area and elongation for 6 and 24 hours after mating were pooled and presented in Table 6.4.

The mean percentage of elongation of spermatozoa from the vagina, cervix, uterus and oviducts were significantly different ($P \leq 0.05$). The biggest percentage of elongation was for anterior vagina spermatozoa followed by spermatozoa from the cervix, uterus and oviducts (Table 6.4). The head area of spermatozoa from the oviducts was significantly smaller ($P \leq 0.05$) than those in the uterus, cervix and vagina.

Table 6.4 Mean (± SEM) elongation and head area of spermatozoa at different sites of the reproductive tract of ewes.

Site in reproductive tract	Elongation (%)*	Head area (μm^2)*
Anterior vagina	44.5 ± 4.5 [a]	2.8 ± 0.4 [a]
Posterior cervix	42.5 ± 2.6 [a]	2.5 ± 0.2 [a]
Mid-cervix	43.2 ± 2.1 [a]	2.7 ± 0.3 [a]
Anterior cervix	43.5 ± 1.2 [a]	2.6 ± 0.2 [a]
Body of uterus	38.5 ± 0.3 [b]	2.5 ± 0.0 [a]
Mid-uterus-right	35.5 ± 7.5 [b]	2.8 ± 0.6 [a]
Anterior uterus-right	38.3 ± 6.2 [b]	2.9 ± 0.3 [a]
Mid-uterus-left	37.2 ± 7.2 [b]	2.9 ± 0.3 [a]
Anterior uterus-left	37.6 ± 3.4 [b]	2.7 ± 0.1 [a]
Right isthmus	32.0 ± 0.4 [c]	2.3 ± 0.1 [b]
Right ampulla	30.8 ± 0.5 [c]	2.0 ± 0.2 [b]
Left isthmus	31.8 ± 0.0 [c]	2.2 ± 0.1 [b]
Left ampulla	30.2 ± 0.4 [c]	1.8 ± 0.1 [b]

*Pooled data for ewes 6 and 24 hours after mating. Data with different superscripts within each column were significantly different ($P \leq 0.05$).

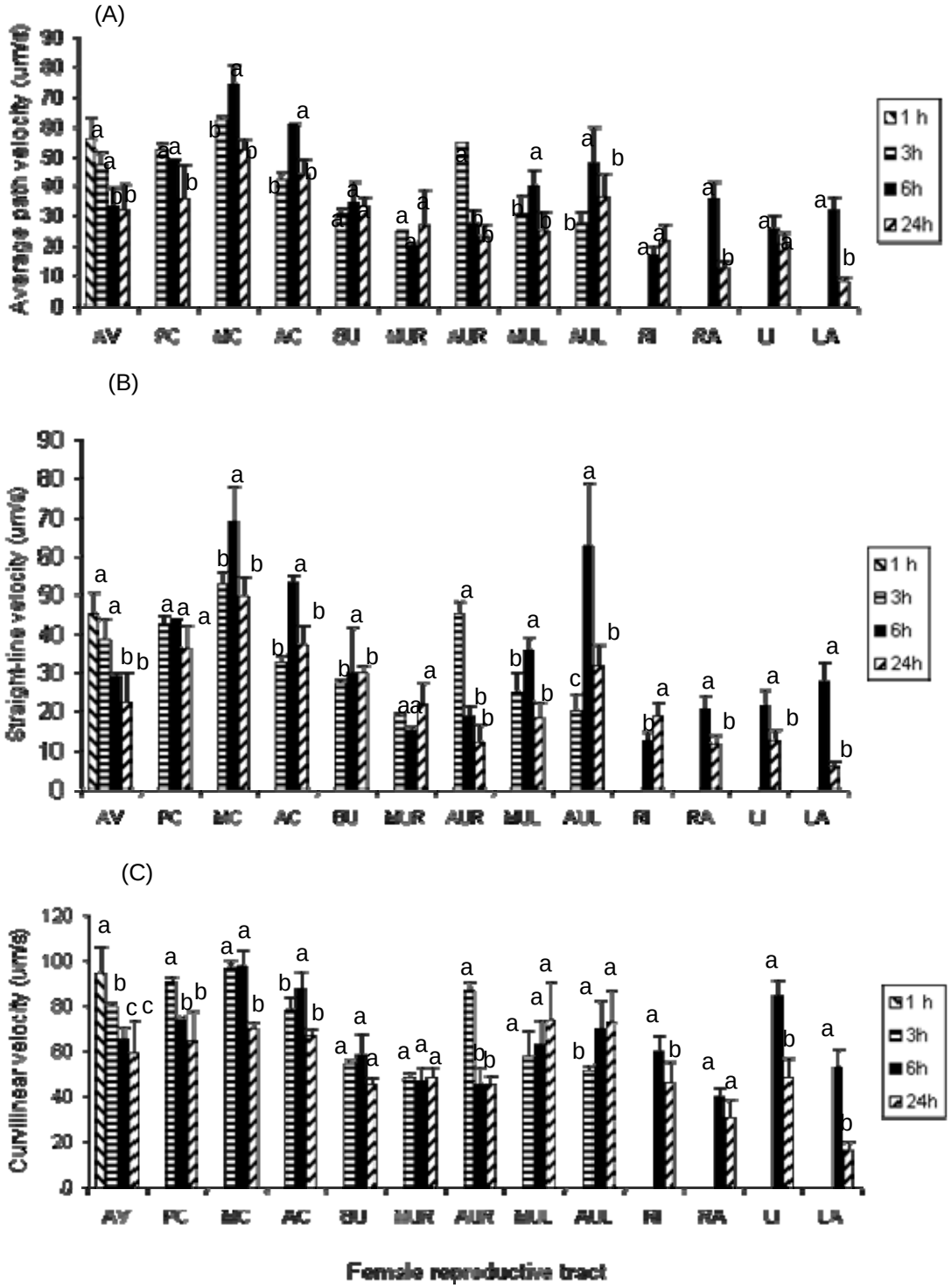


Figure 6.3 The mean (± SEM) average path velocity (Figure A), straight-line velocity (Figure B) and curvilinear velocity (Figure C) of spermatozoa in the anterior vagina, cervix, uterus and oviducts of ewes at 1, 3, 6 and 24 hours after mating. Different letters above bars indicate significant differences ($P \leq 0.05$) within that AV = anterior vagina; PC, MC, AC = posterior, mid, anterior cervix; BU = body of uterus; MUR, AUR = mid, anterior uterus-right; MUL, AUL = mid, anterior uterus-left; RI, LI = right, left isthmus; RA, LA = right, left ampulla.

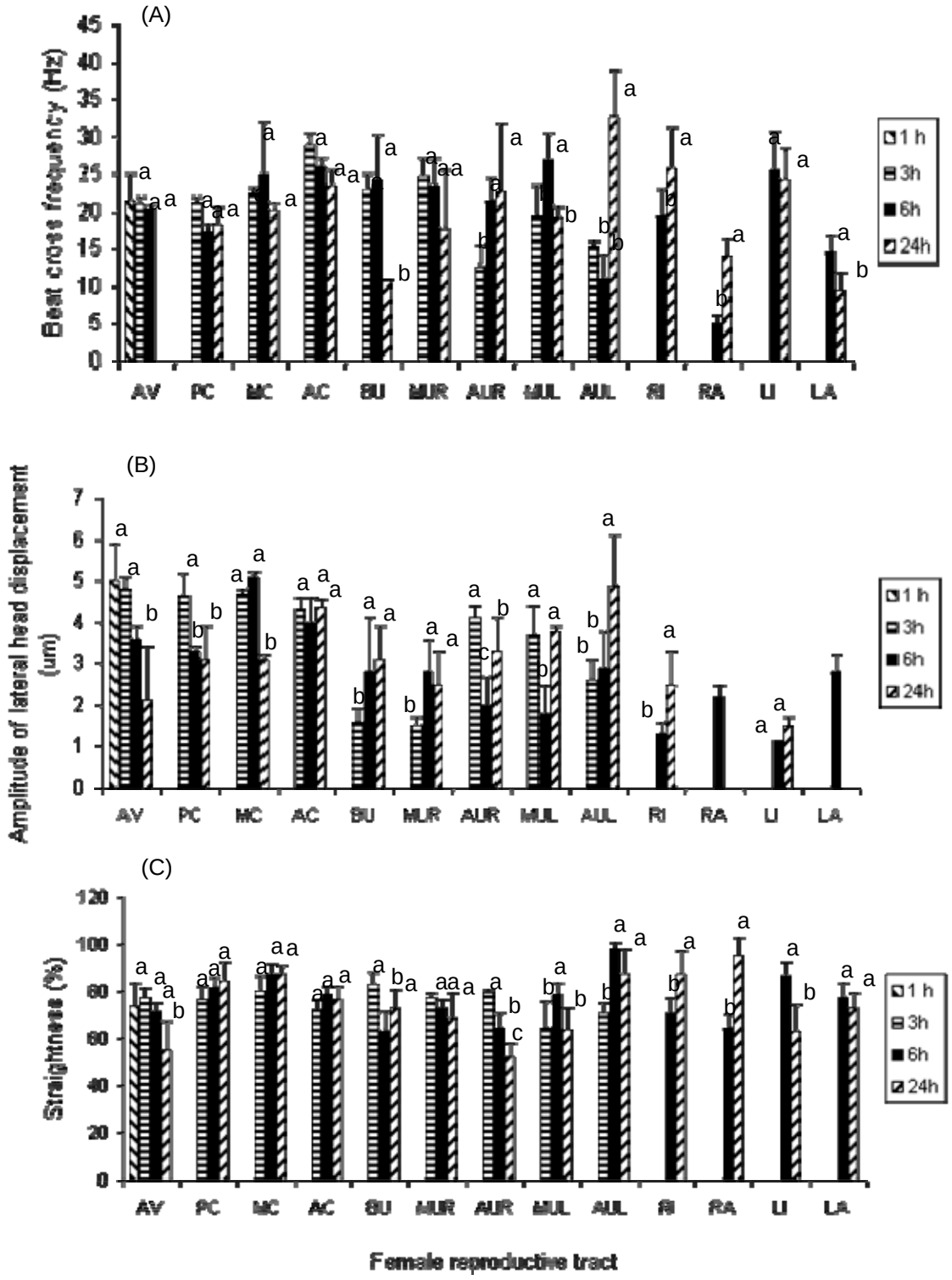


Figure 6.4 The mean (± SEM) beat cross frequency (Figure A), amplitude of lateral head displacement (Figure B) and straightness (Figure C) of spermatozoa in the anterior vagina, cervix, uterus and oviducts of ewes at 1, 3, 6 and 24 hours after mating. Different letters above bars indicate significant differences. BU = body of uterus; MUR, AUR = mid, anterior uterus-right; MUL, AUL = mid, anterior uterus-left; RI, LI = right, left isthmus; RA, LA = right, left ampulla.

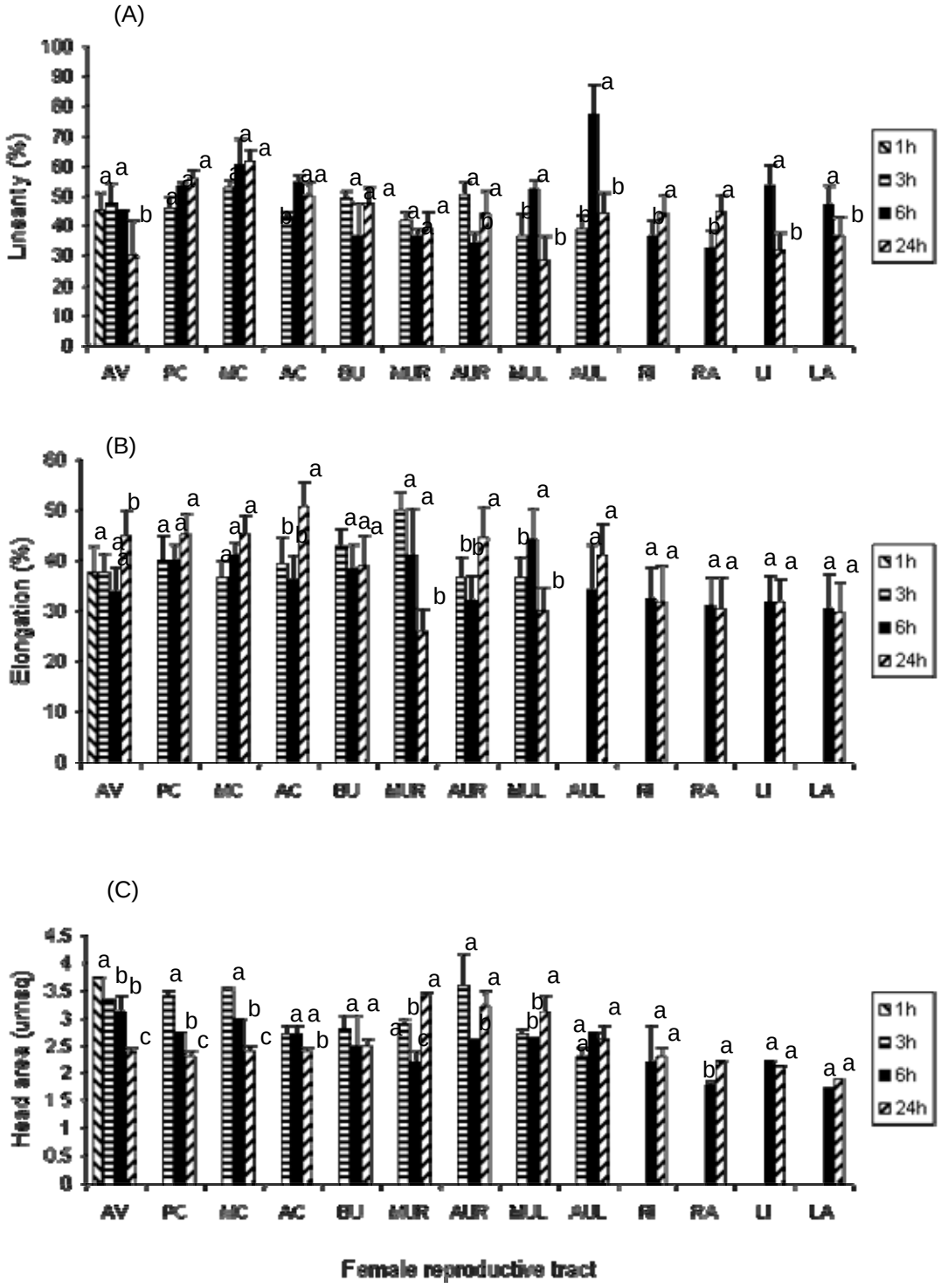


Figure 6.5 The mean (± SEM) linearity (Figure A), elongation (Figure B) and head area (Figure C) of spermatozoa in the anterior vagina, cervix, uterus and oviducts of ewes at 1, 3, 6 and 24 hours after mating. Different letters above bars indicate significant differences ($P \leq 0.05$) within that part of the female reproductive tract. AV = anterior vagina; PC, MC, AC = posterior, mid, anterior cervix;BU = body of uterus; MUR, AUR = mid, anterior uterus-right; MUL, AUL = mid, anterior uterus-left; RI, LI = right, left isthmus; RA, LA = right, left ampulla.

6.3.5 Velocity of spermatozoa in the ipsilateral and contralateral anterior uterus and oviducts

The VAP, VSL and VCL of spermatozoa in the anterior uterus, isthmus and ampulla of the ipsilateral side to the ovary bearing the pre-ovulatory follicle or corpus haemorrhagicum were significantly faster than in the contralateral side at six hours after mating (Figure 6.6). However, at 24 hours after mating, spermatozoa in the mid-uterus and isthmus of the contralateral side had a VAP, VSL and VCL faster than in the ipsilateral side (Figure 6.7). No spermatozoa were present in the ipsilateral anterior uterus or the contralateral ampulla.

6.3.6 The dimensions of spermatozoa in Merino ram semen

While there was no significant difference in the overall length of spermatozoa, there were significant differences in the width and length of the head of spermatozoa (Table 6.5). Two rams (R9 and R13) had narrower and shorter spermatozoal heads than the other four rams. At the other extreme was R12 which had wider and longer heads and a trend to have spermatozoa whose overall length was greater than spermatozoa from the other rams. This was consistent with data presented in Chapter 4 (Section 4.3.2) where the head area of spermatozoa of R12 was significantly greater than R9, R13 and R16.

Table 6.5 The mean (± SEM) dimensions of Merino ram spermatozoa.

Ram	N	Head width (μm)	Head length (μm)	Tail length (μm)	Sperm length (μm)
Ram 5	19	5.10 ± 0.04 ^{ab}	7.79 ± 0.02 ^{ab}	58.32 ± 0.54 ^{ab}	66.11 ± 0.55 ^{a}
Ram 9	22	4.87 ± 0.10 ^{b}	7.82 ± 0.02 ^{b}	58.68 ± 0.39 ^{ab}	66.47 ± 0.43 ^{a}
Ram 10	18	5.10 ± 0.48 ^{ab}	7.86 ± 0.03 ^{b}	58.17 ± 0.54 ^{a}	66.05 ± 0.55 ^{a}
Ram 12	25	5.16 ± 0.14 ^{a}	7.93 ± 0.07 ^{a}	59.85 ± 0.69 ^{b}	67.77 ± 0.71 ^{a}
Ram 13	26	4.92 ± 0.58 ^{b}	7.83 ± 0.02 ^{b}	57.93 ± 0.39 ^{a}	65.82 ± 0.40 ^{a}
Ram 16	21	4.99 ± 0.57 ^{ab}	7.82 ± 0.03 ^{b}	58.12 ± 0.59 ^{b}	65.92 ± 0.49 ^{a}

Data with different superscripts within each column were significantly different ($P \leq 0.05$).
N = number of spermatozoa measured.

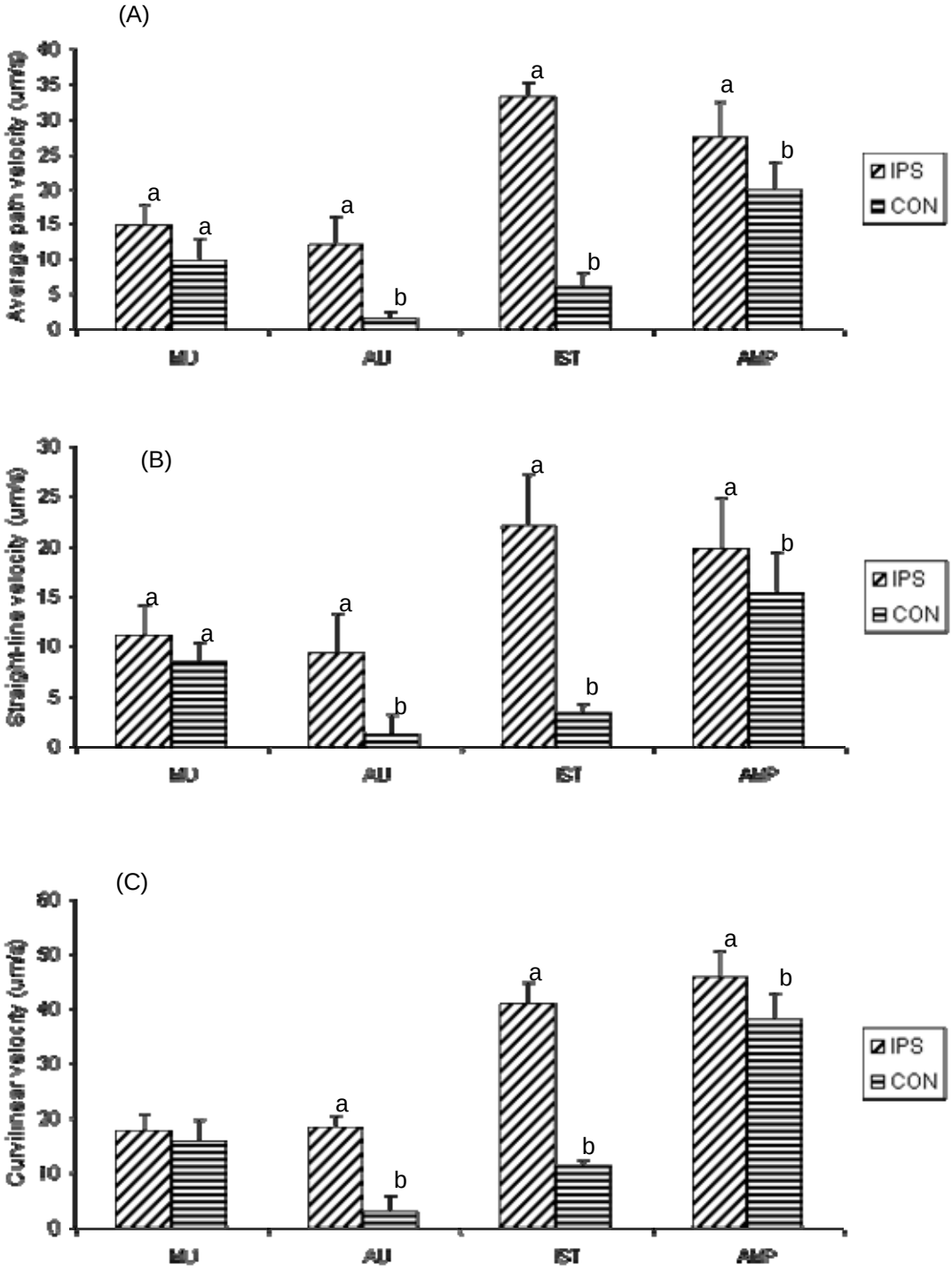


Figure 6.6 The mean (± SEM) average path velocity (Figure A), straight-line velocity (Figure B) and curvilinear velocity (Figure C) of spermatozoa in the ipsilateral and contralateral uterus and oviducts of five ewes six hours after mating. Different letters above bars indicate significant differences ($P \leq 0.05$) within that part of the female reproductive tract.
MU = mid-uterus; AU = anterior uterus; IST = isthmus; AMP = ampulla
IPS = ipsilateral; CON = contralateral.

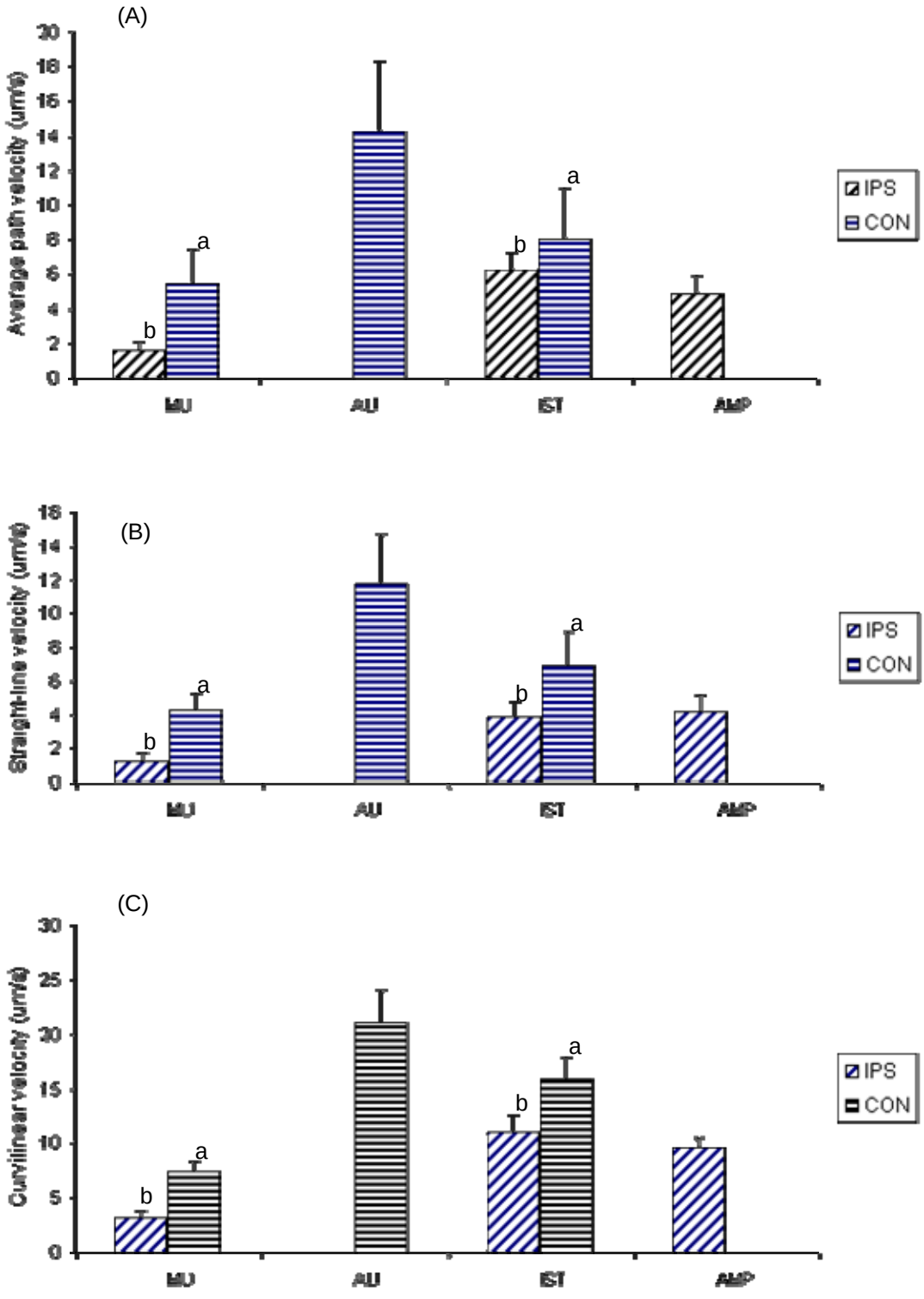


Figure 6.7 The mean (± SEM) average path velocity (Figure A), straight-line velocity (Figure B) and curvilinear velocity (Figure C) of spermatozoa in the ipsilateral and contralateral uterus and oviducts of five ewes 24 hours after mating. Different letters above bars indicate significant differences ($P \leq 0.05$) within MU = mid-uterus; AU = anterior uterus; IST = isthmus; AMP = ampulla IPS = ipsilateral; CON = contralateral.

6.3.7 The dimensions of spermatozoa in the reproductive tract of ewes after natural mating

As it was demonstrated that there were significant differences between rams in the width and length of the head of spermatozoa, the data for the dimensions of spermatozoa in the reproductive tract of ewes was analysed for each ram used (Table 6.6 and 6.7). A consistent feature was that regardless of the site in the female reproductive tract, there was no significant difference in the overall length nor the tail length of spermatozoa. However a consistent feature at both 6 and 24 hours after mating was that spermatozoa in the oviducts had a significantly narrower head ($P \leq 0.05$) than other sites in the reproductive tract. At 24 hours after mating, the spermatozoa in the oviducts had significantly shorter head, a feature that was often observed at 6 hours after mating. No significant difference was found between the isthmus or ampulla or the left or right oviducts.

Table 6.6 The mean (± SEM) dimensions of ram spermatozoa derived from ewes 6 hours after mating.

Site in reproductive tract	(N)	Head width (μm)	Head length (μm)	Tail length (μm)	Sperm length (μm)
Ram 9 x 2 ewes:					
Anterior vagina	(4)	4.94 ± 0.69 [a]	7.84 ± 0.58[a]	58.82 ± 1.24[a]	66.65 ± 1.43[a]
Posterior cervix	(3)	5.00 ± 0.25[a]	7.93 ± 0.35[a]	59.80 ± 0.26[a]	67.72 ± 0.65[a]
Midcervix	(5)	4.94 ± 0.35[a]	7.86 ± 0.25[a]	59.48 ± 0.65[a]	67.34 ± 0.71[a]
Anterior cervix	(6)	4.94 ± 0.42[a]	7.86 ± 0.15[a]	58.37 ± 0.75[a]	66.25 ± 0.24[a]
Body of uterus	(2)	4.85	7.89	58.01	65.90
Miduterine-right	(4)	4.94 ± 0.37[a]	7.90 ± 0.14[a]	58.28 ± 1.16[a]	66.20 ± 1.35[a]
Anterior uterine-right	(6)	4.98 ± 0.32[a]	7.06 ± 0.42[a]	59.33 ± 1.00[a]	66.42 ± 1.53[a]
Miduterine-left	(4)	4.90 ± 0.26[a]	7.80 ± 0.28[a]	57.18 ± 1.03[a]	64.95 ± 1.16[a]
Anterior uterine-left	(5)	4.90 ± 0.18[a]	7.80 ± 0.15[a]	57.80 ± 0.31[a]	65.61 ± 1.41[a]
Right isthmus	(4)	4.66 ± 0.28[b]	7.68 ± 0.33[a]	59.11 ± 0.28[a]	66.76 ± 0.34[a]
Right ampulla	(6)	4.49 ± 0.23[b]	7.26 ± 0.32[b]	59.98 ± 0.23[a]	67.34 ± 0.37[a]
Left isthmus	(3)	4.67 ± 0.15[b]	7.75 ± 0.30[a]	59.53 ± 0.21[a]	67.24 ± 0.36[a]
Left ampulla	(5)	4.62 ± 0.18[b]	7.22 ± 0.32[b]	59.98 ± 0.21[a]	67.32 ± 0.35[a]
Ram 12 x 1 ewe:					
Anterior vagina	(5)	5.15 ± 0.45[a]	7.83 ± 0.46[a]	59.61 ± 0.79[a]	67.42 ± 1.86[a]
Posterior cervix	(3)	5.20 ± 0.12[a]	7.95 ± 0.68[a]	59.71 ± 1.80[a]	67.64 ± 1.83[a]
Midcervix	(6)	5.18 ± 0.25[a]	7.85 ± 0.28[a]	60.15 ± 0.40[a]	68.07 ± 0.57[a]
Anterior cervix	(7)	5.15 ± 0.42[a]	7.95 ± 0.11[a]	59.63 ± 0.62[a]	67.57 ± 0.63[a]
Body of uterus	(3)	5.18 ± 0.54[a]	7.93 ± 0.15[a]	59.83 ± 1.57[a]	67.77 ± 0.60[a]
Miduterine-right	(3)	5.15 ± 0.34[a]	7.93 ± 0.25[a]	59.62 ± 1.68[a]	67.57 ± 1.91[a]
Anterior uterine-right	(5)	5.18 ± 0.23[a]	7.87 ± 0.37[a]	59.08 ± 1.26[a]	66.97 ± 1.67[a]
Miduterine-left	(5)	5.13 ± 0.15[a]	7.83 ± 0.26[a]	59.65 ± 1.32[a]	67.49 ± 1.41[a]
Anterior uterine-left	(4)	5.20 ± 0.12[a]	7.80 ± 0.15[a]	59.82 ± 0.67[a]	67.65 ± 1.21[a]
Right isthmus	(7)	4.81 ± 0.14[b]	7.78 ± 0.45[a]	59.65 ± 0.44[a]	67.46 ± 0.71[a]
Right ampulla	(6)	4.06 ± 0.16[b]	7.44 ± 0.28[b]	59.69 ±0.92[a]	67.11 ± 0.22[a]
Left isthmus	(2)	4.82	7.75	60.12	67.87
Left ampulla	(4)	4.35 ± 0.13[b]	7.47 ± 0.36[b]	58.78 ± 0.58[a]	66.28 ± 0.63[a]

Data with different superscripts within each column were significantly different ($P \leq 0.05$). N is the number of spermatozoa measured.

Table 6.6 (Continued)

Site in reproductive Tract	(N)	Head width (μm)	Head length (μm)	Tail length (μm)	Sperm length (μm)
Ram 13 x 3 ewes:					
Anterior vagina	(4)	5.16 ± 0.29[a]	7.83 ± 0.17[a]	58.32 ± 1.24[a]	66.17 ± 0.85[a]
Posterior cervix	(4)	4.94 ± 0.56[a]	7.69 ± 0.14[a]	59.06 ± 1.06[a]	66.73 ± 0.61[a]
Midcervix	(6)	5.11 ± 0.21[a]	7.85 ± 0.28[a]	58.25 ± 0.65[a]	66.07 ± 0.57[a]
Anterior cervix	(6)	4.83 ± 0.52[a]	7.84 ± 0.98[a]	57.82 ± 0.75[a]	65.68 ± 0.46[a]
Body of uterus	(3)	5.07 ± 0.21[a]	7.80 ± 0.16[a]	57.48 ± 1.53[a]	65.30 ± 1.20[a]
Miduterine-right	(3)	4.93 ± 0.13[a]	7.80 ± 0.16[a]	57.42 ± 1.16[a]	65.20 ± 0.67[a]
Anterior uterine-right	(6)	5.16 ± 0.30[a]	7.69 ± 0.20[a]	58.46 ± 1.12[a]	66.04 ± 1.21[a]
Miduterine-left	(4)	5.20 ± 0.41[a]	7.77 ± 0.25[a]	57.28 ± 1.03[a]	65.07 ± 0.27[a]
Anterior uterine-left	(4)	5.16 ± 0.11[a]	7.70 ± 0.16[a]	58.03 ± 0.22[a]	65.75 ± 0.21[a]
Right isthmus	(5)	4.65 ± 0.12[b]	7.52 ± 0.14[b]	57.80 ± 0.21[a]	65.32 ± 0.72[a]
Right ampulla	(7)	4.45 ± 0.15[b]	7.43 ± 0.25[b]	57.80 ± 0.17[a]	65.22 ± 0.62[a]
Left isthmus	(3)	4.59 ± 0.13[b]	7.57 ± 0.13[b]	57.82 ± 0.24[a]	65.39 ± 0.42[a]
Left ampulla	(3)	4.47 ± 0.17[b]	7.45 ± 0.25[b]	58.77 ± 0.34[a]	66.22 ± 0.20[a]
Ram 16 x 2 ewes:					
Anterior vagina	(4)	5.22 ± 0.32[a]	7.83 ± 0.36[a]	58.52 ± 1.79[a]	66.37 ± 1.86[a]
Posterior cervix	(4)	4.97 ± 0.36[a]	7.85 ± 0.18[a]	58.36 ± 0.80[a]	66.23 ± 0.83[a]
Midcervix	(6)	5.38 ± 0.13[a]	7.75 ± 0.28[a]	58.34 ± 0.40[a]	66.07 ± 1.57[a]
Anterior cervix	(6)	4.89 ± 0.13[a]	7.75 ± 0.11[a]	58.53 ± 0.62[a]	66.27 ± 0.63[a]
Body of uterus	(3)	5.10 ± 0.14[a]	7.83 ± 0.15[a]	58.28 ± 1.57[a]	66.13 ± 1.60[a]
Miduterine-right	(3)	5.16 ± 0.09[a]	7.83 ± 0.25[a]	57.84 ± 1.68[a]	65.67 ± 1.91[a]
Anterior uterine-right	(6)	4.89 ± 0.13[a]	7.77 ± 0.37[a]	57.66 ± 1.26[a]	65.45 ± 1.67[a]
Miduterine-left	(3)	4.96 ± 0.18[a]	7.83 ± 0.26[a]	58.36 ± 1.32[a]	66.19 ± 1.41[a]
Anterior uterine-left	(4)	4.88 ± 0.19[a]	7.80 ± 0.15[a]	57.93 ± 0.67[a]	65.75 ± 1.21[a]
Right isthmus	(6)	4.54 ± 0.29[b]	7.58 ± 0.45[a]	57.79 ± 0.65[a]	65.36 ± 0.61[a]
Right ampulla	(6)	4.44 ± 0.29[b]	7.34 ± 0.28[b]	58.75 ±0.76[a]	66.11 ± 0.52[a]
Left isthmus	(3)	4.47 ± 0.36[b]	7.55 ± 0.26[a]	57.87 ± 0.57[a]	65.44 ± 0.39[a]
Left ampulla	(3)	4.44 ± 0.17[b]	7.37 ± 0.36[b]	58.25 ± 0.66[a]	65.63 ± 0.43[a]

Data with different superscripts within each column were significantly different ($P \leq 0.05$). N is the number of spermatozoa measured.

Table 6.7 The mean (± SEM) dimensions of ram spermatozoa derived from mated ewes 24 hours after mating.

Site in reproductive tract	(N)	Head width (μm)	Head length (μm)	Tail length (μm)	Sperm length (μm)
Ram 9 x 2 ewes:					
Anterior vagina	(4)	4.88 ± 0.67[a]	7.80 ± 0.23	59.07 ± 1.24[a]	66.85 ± 1.43[a]
Posterior cervix	(2)	5.05	7.83	58.89	66.72
Midcervix	(4)	5.08 ± 0.15[a]	7.85 ± 0.15[a]	59.38 ± 0.65[a]	67.34 ± 0.41[a]
Anterior cervix	(3)	4.75 ± 0.23[a]	7.78 ± 0.12[a]	59.45 ± 0.75[a]	67.25 ± 0.34[a]
Body of uterus	(2)	4.85	7.84	58.91	66.75
Miduterine-right	(3)	4.88 ± 0.35[a]	7.87 ± 0.14[a]	58.45 ± 0.18[a]	66.32 ± 1.55[a]
Anterior uterine-right	(3)	4.74 ± 0.13[a]	7.78 ± 0.22[a]	59.63 ± 0.56[a]	67.39 ± 0.33[a]
Miduterine-left	(3)	4.86 ± 0.26[a]	7.70 ± 0.16[a]	58.58 ± 0.33[a]	66.31 ± 1.46[a]
Anterior uterine-left	(4)	4.89 ± 0.16[a]	7.80 ± 0.15[a]	58.79 ± 0.13[a]	66.61 ± 0.81[a]
Right isthmus	(5)	4.51 ± 0.27[b]	7.58 ± 0.21[b]	58.52 ± 0.31[a]	66.09 ± 0.34[a]
Right ampulla	(5)	4.46 ± 0.25[b]	7.43 ± 0.14[b]	58.79 ± 0.27[a]	66.18 ± 0.27[a]
Left isthmus	(3)	4.59 ± 0.21[b]	7.45 ± 0.18[b]	58.82 ± 0.25[a]	66.26 ± 0.36[a]
Left ampulla	(6)	4.43 ± 0.16[b]	7.37 ± 0.22[b]	58.78 ± 0.21[a]	66.17 ± 0.22[a]
Ram 12 x 3 ewes:					
Anterior vagina	(3)	5.35 ± 0.44[a]	7.79 ± 0.36[a]	60.13 ± 0.79[a]	67.95 ± 1.78[a]
Posterior cervix	(2)	5.37	7.84	59.90	67.74
Midcervix	(4)	4.95 ± 0.37[a]	7.73 ± 0.23[a]	60.52 ± 0.15[a]	68.28 ± 0.26[a]
Anterior cervix	(2)	5.32	7.84	59.63	67.47
Body of uterus	(2)	5.26	7.86	59.83	67.69
Miduterine-right	(3)	4.98 ± 0.22[a]	7.89 ± 0.25[a]	59.36 ± 1.25[a]	67.21 ± 1.23[a]
Anterior uterine-right	(3)	5.12 ± 0.23[a]	7.90 ± 0.36[a]	59.65 ± 1.26[a]	67.56 ± 1.12[a]
Miduterine-left	(2)	5.34	7.88	59.48	67.36
Anterior uterine-left	(3)	5.33 ± 0.14[a]	7.86 ± 0.27[a]	59.90 ± 0.45[a]	67.78 ± 0.48[a]
Right isthmus	(5)	4.55 ± 0.17[b]	7.36 ± 0.13[b]	59.88 ± 0.42[a]	67.25 ± 0.22[a]
Right ampulla	(6)	4.26 ± 0.14[b]	7.18 ± 0.16[b]	60.21 ± 0.31[a]	67.38 ± 0.34[a]
Left isthmus	(2)	4.45	7.24	60.54	67.78
Left ampulla	(5)	4.31 ± 0.15[b]	7.12 ± 0.11[b]	60.21 ± 0.22[a]	67.35 ± 0.38[a]

Data with different superscripts within each column were significantly different ($P \leq 0.05$). N is the number of spermatozoa measured.

Table 6.7 (Continued)

Site in reproductive Tract	(N)	Head width (μm)	Head length (μm)	Tail length (μm)	Sperm length (μm)
Ram 13 x 1 ewe:					
Anterior vagina	(4)	5.12 ± 0.55[a]	7.78 ± 0.68[a]	57.54 ± 1.24[a]	65.32 ± 1.15[a]
Posterior cervix	(3)	4.92 ± 0.28[a]	7.67 ± 0.58[a]	59.05 ± 1.06[a]	66.76 ± 0.61[a]
Midcervix	(4)	5.22 ± 0.28[a]	7.62 ± 0.45[a]	58.58 ± 0.65[a]	66.22 ± 0.57[a]
Anterior cervix	(3)	4.95 ± 0.35[a]	7.84 ± 0.42[a]	58.08 ± 0.75[a]	65.84 ± 0.46[a]
Body of uterus	(2)	5.22	7.83	57.85	65.68
Miduterine-right	(2)	4.84	7.82	57.53	65.35
Anterior uterine-right	(4)	5.23 ± 0.26[a]	7.85 ± 0.26[a]	57.72 ± 1.23[a]	65.58 ± 1.56[a]
Miduterine-left	(3)	5.25 ± 0.15[a]	7.86 ± 0.25[a]	58.57 ± 1.32[a]	66.45 ± 0.67[a]
Anterior uterine-left	(3)	5.22 ± 0.24[a]	7.84 ± 0.21[a]	57.96 ± 1.12[a]	65.78 ± 0.81[a]
Right isthmus	(4)	4.56 ± 0.21[b]	7.42 ± 0.14[b]	58.48 ± 0.64[a]	65.88 ± 0.62[a]
Right ampulla	(5)	4.46 ± 0.15[b]	7.35 ± 0.16[b]	58.43 ± 0.61[a]	65.76 ± 0.51[a]
Left isthmus	(3)	4.59 ± 0.14[b]	7.28 ± 0.13[b]	58.25 ± 0.54[a]	65.55 ± 0.82[a]
Left ampulla	(6)	4.42 ± 0.12[b]	7.16 ± 0.15[b]	58.08 ± 0.64[a]	65.22 ± 0.68[a]
Ram 16 x 3 ewes:					
Anterior vagina	(4)	5.23 ± 0.22[a]	7.85 ± 0.46[a]	58.15 ± 1.79[a]	65.98 ± 1.86[a]
Posterior cervix	(2)	4.94	7.82	57.93	65.75
Midcervix	(3)	5.17 ± 0.16[a]	7.76 ± 0.18[a]	58.28 ± 0.40[a]	66.02 ± 0.57[a]
Anterior cervix	(3)	4.94 ± 0.14[a]	7.62 ± 0.31[a]	57.95 ± 0.62[a]	65.58 ± 0.63[a]
Body of uterus	(3)	5.23 ± 0.12[a]	7.78 ± 0.15[a]	58.03 ± 1.57[a]	65.83 ± 1.25[a]
Miduterine-right	(2)	4.96	7.88	58.60	66.48
Anterior uterine-right	(4)	5.03 ± 0.15[a]	7.90 ± 0.17[a]	57.46 ± 1.26[a]	65.35 ± 1.24[a]
Miduterine-left	(2)	5.15	7.85	57.60	65.45
Anterior uterine-left	(3)	5.33 ± 0.17[a]	7.78 ± 0.15[a]	57.73 ± 0.67[a]	65.48 ± 0.35[a]
Right isthmus	(5)	4.65 ± 0.18[b]	7.54 ± 0.15[b]	58.66 ± 0.44[a]	66.22 ± 0.42[a]
Right ampulla	(5)	4.48 ± 0.17[b]	7.48 ± 0.18[b]	58.25 ±0.69[a]	65.75 ± 0.38[a]
Left isthmus	(2)	4.67	7.56	58.27	65.83
Left ampulla	(5)	4.46 ± 0.16[b]	7.42 ± 0.16[b]	57.83 ± 0.58[a]	65.23 ± 0.48[a]

Data with different superscripts within each column were significantly different ($P \leq 0.05$). N is the number of spermatozoa measured.

6.4 Discussion

Consistent with previous reports in the literature (Quinlivan and Robinson, 1969; Mattner and Braden, 1969b; Hawk and Cooper, 1977), there was a dramatic reduction in the recovery of spermatozoa from the uterine horns and oviducts of ewes compared with the cervix and vagina. The recovery was also influenced by time after mating with the recovery from the uterus and oviducts at 24 hours being significantly less than at 3 and 6 hours. On the other hand, the recovery from the cervix, at least in the anterior and mid-cervix was similar at 6 and 24 hours after mating but significantly less than at 3 hours after mating adding support to the view that spermatozoa survive longer in the cervix and the cervix is a reservoir of spermatozoa (Mattner, 1966; Mattner and Braden 1969; Hawk, 1983).

An unexpected finding was the low percentage of motile spermatozoa in the uterus and oviducts at 3 and 6 hours after mating. It was expected that as it takes 6-8 hours (Hunter *et al*., 1980) for a fertilising population of spermatozoa to reach the oviducts that a much higher percentage of motile spermatozoa would have been present at 3 and 6 hours after mating in the uterus and the oviducts.

There might have been an influence of the experimental technique on the motility results. Samples were always collected and analysed for motility characteristics in the order of vagina first and the oviducts last and thus there was a time difference in obtaining the data. The average interval between analysing the first and last sample with the CASA was 70 ± 12 minutes. This means that the oviduct samples were stored in the TALP medium at 39 ^{o}C for about 70 minutes after collection from the reproductive tract. While there is some reduction in the motility of spermatozoa incubated in HSOF medium (about 10% after one hour incubation of semen diluted 1:15; see Figure 4.2), and the motility of spermatozoa in HSOF medium and in TALP medium was not significantly different (see Table 4.3), it would seem that the technique used was not responsible for the low percentage of motile spermatozoa recorded.

The design of the experiment could be criticised because the ewes were not inseminated with exactly the same number of spermatozoa from the same ram. Artificial insemination with a constant number of spermatozoa from the same ram

was not chosen for a number of reasons. It was believed that in order to study the distribution of spermatozoa in the female reproductive tract it was essential to mate ewes naturally in order to allow for any effects of the mating process on the contractions of the reproductive tract of the ewe (Gilbert *et al.,* 1991). In addition, if there was any component of ram semen such as prostaglandins (Evans and Maxwell, 1987) that might have an effect on distribution of spermatozoa, then these would be likely to be more effective from freshly ejaculated semen rather than semen collected by electroejaculation with subsequent delays before the ewe could be inseminated. There was also a risk in using only one ram for the studies because the ram might become sick or die, become infertile or develop refractiveness to repeated electroejaculation.

The above concerns were addressed by using a one hour mating period and using the same four rams throughout the experiment. Consistency of insemination dose was approximately achieved because the ewes were mated on an average of three times.

An extremely interesting finding was that significantly more spermatozoa were present in the oviduct ipsilateral to the ovary bearing the preovulatory follicle or the corpus haemorrhagicum. Significantly more spermatozoa were also present in the ipsilateral anterior and mid-uterus 24 hours after mating but not 6 hours after mating. This finding is in partial support of the concept proposed by Hunter *et al.* (1983) that the ovary via its hormones influences the function of the oviduct. This work was done in the pig and the authors believed that the ovarian hormones were causing release of spermatozoa from the isthmus allowing progress to the ampulla and subsequently fertilisation.

The most likely explanation for the increased numbers of spermatozoa on the ipsilateral side is that there is an increased concentration of hormones in this uterine horn and oviduct that have originated from the preovulatory follicle and corpus haemorrhagicum. The effect of the hormones could be to increase contractions of the uterus or alter secretory products of the endometrium to facilitate migration of spermatozoa. There might even be substances that specifically attract spermatozoa. There is evidence from work in the cow that there is increased concentration of ovarian hormones on the ipsilateral side, at least in the oviduct (Wijayagunawardane *et al*., 1998). In addition, work reported for the human (Kunz *et al*., 1988) provides

evidence of an increased vascular perfusion of the fundal myometrium ipsilateral to the ovary bearing the preovulatory follicle. Preliminary data suggests there are increased contractions of the ipsilateral oviduct in the cow (Bennet *et al*., 1988). However, what is important to determine in the context of the results in this Chapter is whether there are increased contractions of the ipsilateral uterine horn capable of moving spermatozoa along the uterine horn and to the oviduct.

If there are increased concentrations of ovarian hormones in the ipsilateral oviduct and uterine horn in the ewes, then the hormones could arrive there by either lymphatic or blood vessels. Given the accepted view that there is a countercurrent transfer of prostaglandin $F_2\alpha$ from the uterine vein to the ovarian artery, then it is possible that there could be a countercurrent transfer of hormones from the ovarian vein into an artery that supplies the oviduct and anterior half of the uterine horn. This is what Hunter *et al*. (1983) proposed for the pig although they worked on the basis that the utero-tubal artery derived from the ovarian artery was supplying blood to only the isthmus and utero-tubal junction.

There is a need for more detailed studies on the lymphatic vessels and vascular relationships between the ovary and the oviduct and uterine horns in the ewe if the mechanism influencing distribution of spermatozoa is to be understood. While there have been anatomical studies of extrinsic blood vessels of the ovary of the ewe (Lee and O'Shea, 1976), their report does not completely answer how hormones from the ovarian vein could end up in the tissues of the oviduct and uterus.

In addition to there being more spermatozoa in the ipsilateral oviduct, these spermatozoa were significantly more active at 6 hours after mating as measured by VAP, VSL and VCL. The same significant difference was found in the anterior uterine horn and while the trend was also apperent in the mid-uterine horn, the difference was not statistically significant. These data suggest that at 6 hours after mating the luminal environment of the ipsilateral oviduct and uterus was promoting the activity of spermatozoa. However, the data indicate that the reverse was the case 24 hours after mating.

The spermatozoa that reach the oviducts had heads that were narrower than spermatozoa elsewere in the reproductive tract. However, there was no significant

difference in the overall length of spermatozoa regardless of the anatomical site. This difference between spermatozoa in the oviducts and elsewhere was consistent in all ewes and all the four rams used and was present at both 6 and 24 hours after mating. The overall length of spermatozoa was within the range reported by Cummins and Woodall (1985).

In addition, when the data on elongation and head area of spermatozoa as determined by CASA were pooled for ewes 6 and 24 hours after mating, it was found that the head area of spermatozoa in the oviducts was significantly smaller than elsewhere in the reproductive tract. There was also a distinct gradient in the elongation of spermatozoa from the vagina to the oviducts. This means that the spermatozoa in the vagina had a more rounded head while those in the oviducts with a lower percentage elongation had a narrower and more tapered head.

The significant of these findings can be interpreted in one of two ways. It could be envisaged that spermatozoa that have a narrower head would meet less obstruction to their movement along the reproductive tract and thus are more likely to reach the oviducts. An alternative explanation might be that spermatozoa with a narrower and shorter head have an innate greater velocity, in particular straight-line velocity and thus are more likely to reach the oviducts.

No data were collected on spermatozoa in the oviducts of the eight ewes that were killed three hours after mating. This reason for this was that it was expected, based on the literature (Hunter *et al*., 1980), that spermatozoa capable of fertilising oocytes would not be present in the oviducts three hours after mating. The focus of the research work was the study of those spermatozoa capable of fertilising oocytes. In hindsight and in light of the results obtained this was a mistake. It would be useful to know if those spermatozoa that reach the oviduct soon after mating and believed to have no role in fertilisation and die prematurely (First *et al.,* 1968; Baker and Degen, 1972; Hunter *et al.,* 1980; Anderson, 1991) are in fact all dead by three hours after mating. In addition, it would be interesting to know the dimensions and head area of these spermatozoa. If the above interpretation of why spermatozoa in the oviducts have smaller heads is correct, then it would be expected that spermatozoa in the oviducts soon after mating would not have heads that were smaller than other sites in the reproductive tract.

Another consideration of the fact the spermatozoa in the oviduct have narrower and shorter heads is that rams with spermatozoa with narrower heads might have a higher fertility measured in terms of the percentage of pregnant ewes. If this was case, then measurement of heads of spermatozoa could be one of the selection criteria for selection of rams for high fertility. The data in this Chapter along with data from Chapter 4 showed that one ram (R12) had spermatozoa heads that were significantly greater than the other rams. While all the rams were of proven fertility this was based on mating of a small number of ewes and was insufficient to detect significant differences in pregnancy rates.

In conclusion, the data presented in this Chapter provides evidence that the preovulatory follicle and corpus haemorrhagicum influence the distribution of spermatozoa and that the spermatozoa in the oviducts have narrower and shorter heads than elsewere in the reproductive tract. However, the hypothesis that the velocity and movement characteristics of spermatozoa in the anterior uterus and oviducts were different and better than elsewere in the reproductive tract was not supported by the data.

CHAPTER 7

CAPACITATION STATUS OF SPERMATOZOA AT VARIOUS SITES IN THE REPRODUCTIVE TRACT OF MERINO EWES AFTER NATURAL MATING

7.1 Introduction

Ejaculated spermatozoa cannot immediately bind to an oocyte and fertilise it. They must first undergo a series of biochemical and functional modifications, collectively termed capacitation (Austin, 1951; Chang, 1951). Although the molecular mechanisms of capacitation have not been completely elucidated, studies have demonstrated an involvement of numerous structural and biochemical modifications in spermatozoa, such as removal of seminal plasma proteins and modification of spermatozoa surface molecules (Yanagimachi, 1994), changes in membrane composition and fluidity (Harrison *et al*., 1996; Lin and Kan, 1996), activation of ion channels (Arnoult *et al*., 1996; Florman *et al.,*1998), cytoplasmic alkalinisation (Parrish *et al*., 1989; Vredenburgh-Wilberg and Parrish, 1995), loss of sterols (Cross, 1998, 2003), membrane hyperpolarisation (Zeng *et al*., 1995), elevated concentrations of calcium and cAMP (Baldi *et al*., 1991; Fraser and Dermott, 1992; Visconti *et al.,*1995), protein phosphorylation (Duncan and Fraser, 1993; Furuya *et al*., 1993; Visconti *et al*., 1995; Leclere *et al*., 1996; Pommer and Meyers, 2002; Ecroyd *et al.,* 2003; Pommer *et al.,* 2003), actin polymerization (Brener *et al.,* 2003) and increased intracellular pH (Parrish *et al*., 1989; Cross and Razy-Faulkner, 1997; Nakanishi *et al*., 2001). These modifications result in spermatozoa hyperactivity, ability to bind to the zona pellucida, and an ability to undergo the acrosome reaction (Cross, 1998).

The site in the female reproductive tract where fertilising spermatozoa begin and complete capacitation may vary according to species. In species in which semen is deposited in the uterus during coitus, spermatozoa seem to complete all or most of capacitation in the lower segment of the isthmus where fertilizing spermatozoa are stored (Yanagimachi,1994). Evidence from humans (Gould *et al.,* 1985; Lambert *et al.,* 1985) indicates that capacitation may start while spermatozoa are passing through the cervix and it would be expected that the same would apply in the sheep.

There appears to be no systematic study of the distribution of uncapacitated and capacitated spermatozoa at different sites in the female reproductive tract. The aim of the study described in this Chapter was to determine the effect of time after mating on the capacitation status of spermatozoa and the distribution of capacitated spermatozoa in the reproductive tract of Merino ewes.

7.2 Materials and Methods

7.2.1 Animals

The number of ewes from which spermatozoa were examined for capacitation status in the anterior vagina, cervix, uterus and oviducts at 3, 6 and 24 hours after mating are listed in Table 7.1. The management of the ewes and methods for the control of oestrus and mating have been described in Sections 3.1.2 and 3.7. After mating, samples were collected from the female reproductive tract of ewes as described in Section 3.8.

Table 7.1 The number of ewes from which samples of spermatozoa were collected to determine the capacitation status.

Time after mating (hours)	Number of ewes			
	Anterior vagina	Cervix	Uterus	Oviducts
3	23	8	8	0
6	20	14	14	8
24	13	13	13	9

7.2.2 Identification of capacitation status

The capacitation status of spermatozoa was determined as described in Section 3.9. The mean number and range of spermatozoa examined for capacitation status is shown in Table 7.2.

Table 7.2 The mean (± SEM) number and range of spermatozoa examined for capacitation status at different sites in the reproductive tract of ewes at 3, 6 and 24 hours after mating.

Site	Number of spermatozoa		
	3 hours	6 hours	24 hours
Anterior vagina	97.7 ± 3.3	95 ± 9	58.8 ± 18
	(92 - 100)	(75 - 100)	(44 - 84)
Cervix	273 ± 60	272 ± 39	121 ± 37
	(196 - 355)	(221 - 314)	(67 - 164)
Uterine body	50 ± 26	46 ± 9	8.7 ± 4.3
	(27 - 86)	(36 - 59)	(1 - 16)
Miduterus	141 ± 40	21 ± 9.3	7.6 ± 4.6
	(101 - 181)	(15 - 39)	(4 - 18)
Anterior uterus	111 ± 59	109 ± 3.5	3.1 ± 1.1
	(52 - 170)	(105 - 112)	(1 - 6)
Isthmus	-	7.3 ± 6.7	3.2 ± 1.4
		(3 - 23)	(1 - 6)
Ampulla	-	9.6 ± 12	4 ± 1
		(2 - 41)	(1 - 6)

Range is in parenthesis; - = no data.

7.2.3 Statistical analyses

All data were analysed using SPSS software program (SPSS 11.0 Brief Guide, New Jersey, USA). Analysis of variance (ANOVA) one way classification was used to determine the effect of time after mating on the capacitation status. The differences between means were tested by the Least Significant Difference test. Student t-test was used to determine the effect of the ipsilateral side and contralateral side on the capacitation status. Results were considered significantly different when $P \leq 0.05$.

7.3 Results

7.3.1 *In vivo* capacitation status in the reproductive tract of ewes

The capacitation status of spermatozoa in the female reproductive tract was significantly influenced by the time after mating but there was no ram effect. The percentage of capacitated acrosome-intact and acrosome-reacted spermatozoa increased ($P \leq 0.05$) with time after mating. The mean percentage of uncapacitated spermatozoa at 3 hours after mating in the anterior vagina was 70.5% and 72.7% in the posterior cervix decreasing to about 20% in the anterior uterus (Figure 7.1 A). At 6 hours after mating, between about 10% of spermatozoa in the vagina were uncapacitated and about 25% of spermatozoa in the cervix and uterus were uncapacitated whereas the percentage of uncapacitated spermatozoa in the oviducts was down to about 12.5% in the isthmus and 6.5% in the ampulla. All of the capacitated spermatozoa in the oviducts at 6 hours after mating were acrosome-reacted and none had an intact acrosome (Figure 7.1 B). By 24 hours after mating, all or almost all spermatozoa were capacitated and acrosome-reacted (Figure 7.1 C).

The number of capacitated spermatozoa in the uterine horn and oviducts 6 hours after mating was examined for any influences of the side of the pre-ovulatory follicle on capacitation status (Table 7.3). These data were from eight ewes from which spermatozoa from the oviducts and uterine horn were collected and the six ewes from which spermatozoa were collected from the mid and anterior uterine horns. Significantly more capacitated acrosome-reacted spermatozoa were present in the ipsilateral oviduct and uterine horns than the contralateral side but there was no significant effect on the percentage of capacitated acrosome-intact spermatozoa. Taking the results together, significantly more capacitated spermatozoa were found in the ipsilateral side.

Table 7.3 The percentage of capacitated spermatozoa in the uterine horn and oviducts ipsilateral and contralateral to the ovary bearing the preovulatory follicle 6 hours after mating.

Site	Number of spermatozoa examined	Ipsilateral side		Contralateral side	
		CAI	CAR	CAI	CAR
Miduterus	210	9.1 [a]	51.4 [c]	3.8 [b]	35.7 [d]
Anterior uterus	171	7.0 [a]	53.2 [c]	4.7 [b]	35.1 [d]
Isthmus	51	-	54.9 [c]	-	45.1 [d]
Ampulla	77	-	61.0 [c]	-	39.0 [d]

CAI = capacitated acrosome-intact, CAR = capacitated acrosome-reacted, - = no data
Data with different [ab]superscripts within a row were not significantly different ($P > 0.05$); data with [cd]superscripts within row were significantly different ($P \leq 0.05$).

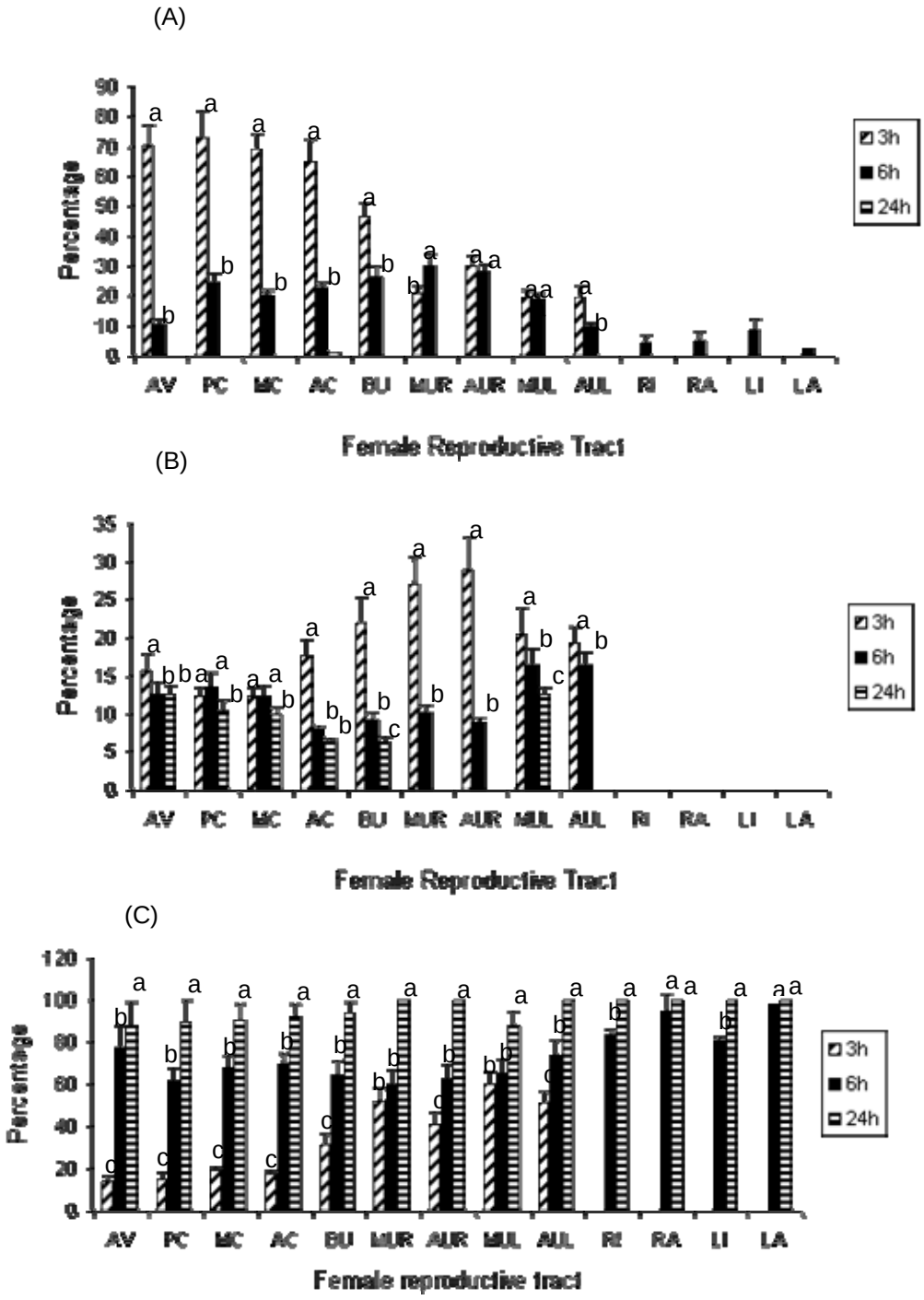


Figure 7.1 The mean (± SEM) percentage of uncapacitated (Figure A), capacitated acrosome-intact (Figure B) and capacitated acrosome-reacted (Figure C) spermatozoa in the anterior vagina, cervix, uterus and oviducts of ewes at 3, 6 and 24 hours after mating.Different letters above bars indicate significant differences ($P \leq 0.05$) within that part of the reproductive tract. Capacitation status in the oviducts was only determined at 6 hours and 24 hours after mating. AV = anterior vagina; PC, MC, AC = posterior, mid, anterior cervix; BU = body of uterus; MUR, AUR = mid, anterior uterus-right; MUL, AUL = mid, anterior uterus-left; RI, LI = right, left isthmus; RA, LA = right, left ampulla.

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7.4 Discussion

The results in this Chapter show that spermatozoa can become capacitated and undergo the acrosome reaction at all sites of the reproductive tract of the ewe. This is the first study that has examined the capacitation status at various sites in the female reproductive tract as well as examining the influence of time after mating on the incidence of capacitation. Other studies of capacitation *in vivo* have been confined to just the uterus or oviduct (Bedford, 1969; Bedford, 1970) and have not looked at the influence of time after mating on capacitation.

A higher percentage of spermatozoa were capacitated in the uterus than the cervix or vagina at three hours after mating. This suggestsed that either the environment in the uterus was more conductive to capacitation than the vagina or cervix or that as spermatozoa were passing through the cervix, they were primed to be more effectively capacitated by the uterine environment. This priming process could be similar to the proposal put forword by Katz *et al*.(1989) that there is a "rubbing off " of material that has been absorbed onto the surface of spermatozoa including seminal plasma proteins as spermatozoa make contact with strands of cervical mucus and that this enables the spermatozoa to become capacitated.

About half of the spermatozoa in the mid and anterior horns at three hours after mating were acrosome-reacted. An interpretation of this observation is that spermatozoa do not need to be exposed to the oviductal environment to complete capacitation and become acrosome-reacted. Given that acrosome-reacted spermatozoa have a short life, then it would be expected that these spermatozoa would not be involved in fertilisation as it would be another 20-24 hours before ovulation would have occurred.

Spermatozoa from the oviducts were not examined at three hours after mating because based on the literature (Hunter *et al.,* 1980; Hunter and Nichol, 1983) it was not expected that many spermatozoa would be present and if present, they would have no role in fertilisation. In hindsight, this was probably a fault in the experimental design and it would have been interesting to see if in fact those spermatozoa that had arrived rapidly soon after mating could be capacitated.

By six hours after mating, between about 75% and 90% spermatozoa in the anterior vagina, cervix and uterus were capacitated of which the majority were acrosome-reacted and by 24 hours after mating virtually all were capacitated. The rate of capacitation in the oviducts seemed to be faster at six hours and depending on the oviductal site up to about 90% were capacitated acrosome-reacted. However, limited confidence can be placed on these values as the numbers of spermatozoa from the oviducts available for evaluation of capacitation status was much less than the numbers from other sites of the reproductive tract. It would be expected however that the capacitation process would be faster in the oviduct in order to provide sufficient numbers of recently capacitated spermatozoa at ovulation.

Whether the capacitated spermatozoa in the vagina, cervix and uterus are able to make any significant movement towards the oviducts is not known. The evidence from studies in the hamster (Shalgi *et al*., 1992) would indicate that capacitated spermatozoa cannot move forward efficiently and in particular have reduced ability to pass through the utero-tubal junction.

The finding that at 6 hours after mating more spermatozoa in the uterine horn and oviduct ipsilateral to the ovary bearing the preovulatory follicle were capacitated further strengthens the observations in Chapter 6 that the ovary was exerting an influence on spermatozoa. The most likely explanation is that hormones from the pre-ovulatory follicle pass to the ipsilateral uterine horn and oviduct via the local vasculture and / or lymphatics and act on the secretory epithelium to the make the ipsilateral uterine and oviduct luminal environment more conducive to capacitation than the contralateral side.

The results of this study confirm the observation made 30 years ago that capacitation *in vivo* occurs quickly in the ewe (Austin, 1974). However, the question does arise as to why all spermatozoa have not undergone capacitation by three or six hours after mating as all the spermatozoa are exposed to the same environment. One interpretation might be that as the ejaculate contains a heterogenous population of spermatozoa with respect to the time they have spent in the cauda epididymidis, it is only the spermatozoa that have spent a longer period in the epididymis and thus can be considered older than spermatozoa that have recently arrived in the cauda

epididymidis that become capacitated first. Alternatively, there is a selection process operating in the female reproductive tract that restrains the capacitation process in the “fitter” spermatozoa until they reach the anterior uterus or oviducts.

CHAPTER 8

GENERAL DISCUSSION

8.1 Scope of the Research Work

The central theme of the research reported in this thesis was to understand what was different about the spermatozoa that reached the anterior uterus and oviducts compared with spermatozoa in other parts of the female reproductive tract. The focus was to measure a range of velocity and movement features as well as morphological features such as head area and capacitation status to identify any distinguishing differences between spermatozoa at different sites of the female reproductive tract.

Sheep were chosen as the study model for several reasons. Firstly, well established methods and management practices were available for the control of oestrous cycles of ewes as well as the collection of semen. Secondly, Merino ewes in north Queensland are usually mono-ovulatory, a point that was necessary in order to study any influence of the ovary bearing the preovulatory follicle on the distribution and function of spermatozoa. Thirdly, ewes have a large enough reproductive tract to enable collection of samples from multiple sites. In the initial planning of the project it was the intention to use surgical procedures to collect samples but this was found to be unsuccessful and impractical particularly when trying to collect samples from the body of the uterus and cervix. Cattle could have been used for the research work but the cost would have been prohibitive.

The studies in Chapter 4 and 5 established the techniques for determining capacitation status of spermatozoa as well as the development of techniques for determining the velocity and movement of spermatozoa using the CASA. It was important to know if there was a significant variation between and within rams as well as any influence of the time of the year on semen characteristics. It was known from previous studies at the Australian Institute of Tropical Veterinary and Animal Science, James Cook University that Merino rams and ewes will breed all the year round, a feature that is different to temperate areas of Australian where sheep breed mainly during autumn.

Two interesting findings emerge from the studies in Chapter 4 and 5. These were that there were differences between rams in the ability of spermatozoa to maintain motility, and by inference survival, in undiluted semen and differences in the capacitation profile of spermatozoa during capacitation *in vitro.* Whether these differences are reflected in the fertility of rams is unknown. The second interesting finding and reported in Chapter 4 was that there were significant differences between rams in the head area of spermatozoa.

The results from the manual measurements of spermatozoa that were collected during studies in Chapter 4 were reported in Chapter 6. This appears to be the first report of differences in head dimensions and head area of spermatozoa within a species and as discussed later, the implications of this finding in the selection of rams for fertility needs detailed investigation. Thurston *et al*. (1999) studied the detailed morphology of spermatozoa from boars and while they identified differences between boars in the length of spermatozoa, they did not identify differences in head width or length.

The key and most significant results in the context of the working hypothesis of this thesis are found in Chapter 6. While the data did not support the hypothesis that spermatozoa in the anterior uterus and oviduct had velocity and movement characteristics that were better than the posterior reproductive tract, two other important findings did emerge. The first was that there was an influence of the ovary containing the pre-ovulatory follicle whereby more spermatozoa were found in the anterior uterus and oviduct ipsilateral to the ovary bearing the pre-ovulatory follicle or corpus haemorrhagicum. In addition, the spermatozoa on the ipsilateral side had a significantly better velocity than the contralateral side but only at 6 hours after mating. The second important finding was that spermatozoa in both the ipsilateral and contralateral oviducts had a narrower and shorter head, a feature that consistently occurred regardless of which ram was used. The data on elongation of spermatozoa showed a very distinct gradient from the vagina through to the oviducts although a distinct gradient was not evident in the data on head area. Possible explanations of the significance and reasons for these findings are given in the Discussion section of Chapter 6. The next section suggests research that should be undertaken to understand how these events have occurred.

There is a vast body of literature on capacitation of mammalian spermatozoa but surprising little detail on capacitation at various sites of the female reproductive tract. The studies in Chapter 7 were an attempt to find out more about capacitation *in vivo.* Much of the research reported in the literature has been on capacitation in the oviducts and the acrosome reaction induced by close association with the oocyte (McNutt and Killian, 1991; Chavarria and Reyes. 1996; Florman *et al*., 1998; Arnoult *et al.,* 1999; Fazeli *et al*., 1999) but the results in Chapter 7 show that capacitation and the acrosome reaction will occur in all sites of the female reproductive tract.

Acrosome-reacted spermatozoa have a very short life span of several minutes (Yanagimachi, 1994) and therefore it is highly unlikely that acrosome-reacted spermatozoa in the posterior half of the reproductive tract could fertilise an oocyte. It also calls into question the role of the so-called sperm reservoir in the cervix (Mattner, 1966) particularly when only about 20% of spermatozoa in the cervix are motile 6 hours after mating and considerably less are motile 24 hours after mating.

In hindsight and in light of the experienced gained, there are several aspects of the research that could be improved or changed. The interval between collection of sample from the ewes and analysis should be reduced. The interval could be reduced by having more than two people doing the collection of samples and another person who could perform the CASA analysis while samples were being collected. In the study, collections were always done in a set order of vagina first and oviducts last. Probably it would have been better to have a random order of collection sites for each ewe.

As explained in Chapter 6, an apportunity was missed to study spermatozoa from the oviducts soon after mating by not collecting oviductal spermatozoa three hours after mating. It would also be useful to have data on spermatozoa closer to the time of ovulation say 12 – 15 hours after mating. This would pose same logistical difficulties although ideally matings could be done at 5.00 – 6.00 pm and collections from the mated ewe done early the following morning. The time of ovulation in north Queensland Merino ewes relative to the end of oestrus has not been accurately determined although in these studies all ewes killed 24 hours after mating in the

morning following onset of oestrus the previous afternoon had ovulated. The period of oestrus in these studies ranged from 24 to 39 hours.

8.2 Future Research Directions

It is often the case that the data obtained from a research project will generate more questions than they answer. This is the case with this thesis. A number of interesting questions have been identified from the work that require in depth study if a comprehensive understanding of the biology of spermatozoa in the female reproductive tract is to be obtained. Some of the questions might have important practical outcomes for the efficient breeding of farm animals.

1. There is a need to define the anatomical relationship of the lymphatic and blood vessels of the ovary, oviducts and anterior half of the uterine horn in the ewe. Once this has been undertaken, the concentration of ovarian hormones in particular oestradiol, progesterone, oxytocin, prostaglandins and endothelin-1 in blood (most likely arterial) and lymph should be determined and a comparison made between the ipsilateral and contralateral side to the ovary bearing the pre-ovulatory follicle.

   Ideally, the studies should use an in-dwelling cannulation system so that repeated blood and/or lymph samples can be collected and a profile of hormone concentrations determined over the peri-ovulatory period. The procedures are likely to be technically difficult because it is expected the blood and lymphatic vessels would be of small diameter making it difficult to surgically install cannulae and to maintain patency. It will be important for collections to be sustained for several days on both the ipsilateral and contralateral sides to the ovary bearing the preovulatory follicle or corpus haemorrhagicum in the same animal to enable meaningful comparison of results.

   An option might be to transfer the studies to the cow where it can be expected that the diameter of the vasculature would be larger. Techniques may be able to be adapted from those described by Hein *et al.* (1988) for the collection of lymph from the ovary and uterus of cows during pregnancy.

2. The frequency and amplitude of uterine contractions of the anterior half of the uterine horns during the peri-ovulatory period needs to be examined on the ipsilateral and contralateral sides to see if this could be the explanation for the difference in the distribution of spermatozoa between the ipsilateral and contralateral sides. This would require the surgical placement of microtransducers in the uterine lumen to detect changes in the internal diameter or the placement of microtransducers in the myometrium to detect myometrial contractions.

3. On the assumption that hormone concentrations in blood supplying the ipsilateral anterior uterus are greater than the contralateral uterine horn then detailed analysis of the composition of endometrial secretions would be required. Ideally, repeated samples should be collected from the ipsilateral and contralateral side of the same animal to enable a accurate and comparative profile to be developed. This would require a chronic in-dwelling canulation system and it is possible that techniques could be developed from the systems described by Gerena and Killian (1990) for the chronic collection of oviduct fluid from cattle.

   A difficulty will be to know what endometrial products to measure and how to determine quantifiable differences between the uterine horns. Endometrial secretions in other species contain many constituents and there is little guidance from the literature on peri-ovulatory endometrium secretions in the ewe. Much of the information in the literature relates to endometrial secretions during the period of maternal recognition of pregnancy and in later pregnancy (see Roberts and Bazer, 1988; Gray *et al.,* 2002).

   If a specific endometrial secretory product or products can be found in higher amounts on the ipsilateral side, the next step will be to determine the effect of these products on the motility and velocity characteristics of spermatozoa as well as if the products can specifically attract spermatozoa.

   The studies on endometrial secretions could be supplemented with studies on differential gene expression in uterine epithelium between the ipsilateral and contralateral side. However, this work will be impeded by the limited progress in the defintion of the ovine genome map (Cockett, 2003).

4. The significance of differences in the dimensions and head area of spermatozoa on fertility of rams needs to be investigated. Initial studies should be conducted on a large number of normal rams (at least 100 is suggested) to gather information on the range in dimensions and head area of spermatozoa. A trial could be devised whereby rams are identified as having either a large or small head area and then used for mating. It is expected that large numbers of ewes would be required to establish statistically significant differences in pregnancy rates. Depending on the number of rams in the large and small head groups, there might be difficulties in having appropriate ram to ewe ratios and it may be necessary to resort to artificial insemination.

8.3 Conclusion

In conclusion, this research work reported in this thesis has examined the question of whether the spermatozoa that reached the anterior uterus and oviducts had had identifiable differences to those in the cervix and posterior uterus. While differences in velocity and movement characteristics were not identified as hypothesised, more spermatozoa were present in the oviducts and anterior uterus ipsilateral to the ovary bearing the pre-ovulatory follicle and corpus haemorrhagicum. These spermatozoa had smaller and narrower heads and better velocity than those on the contralateral side.

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